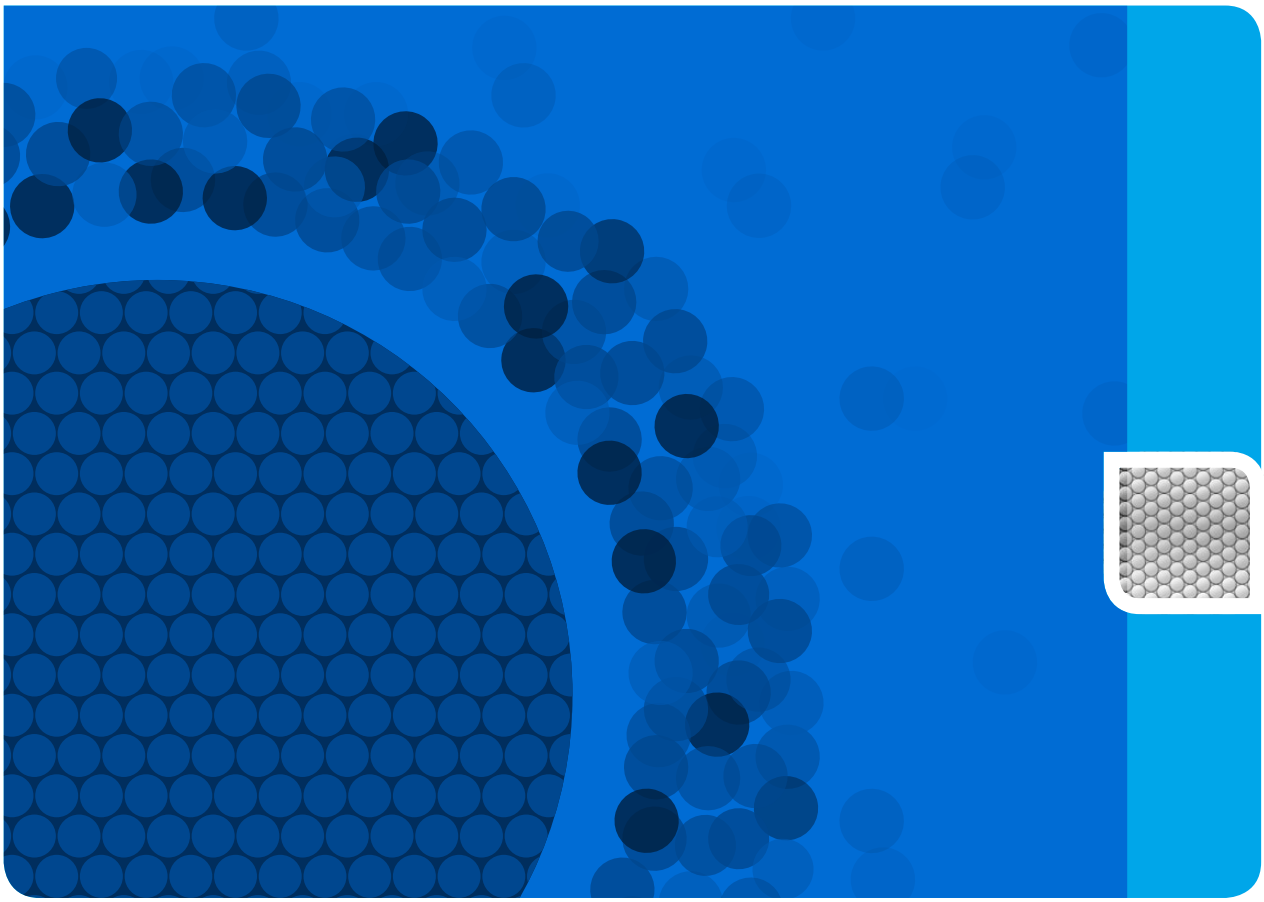

Droplet Digital™ PCR
Applications Guide

BIO-RAD

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1 Droplet Digital™ PCR

Introduction

Droplet Digital polymerase chain reaction (ddPCR™) was developed to provide high-precision, absolute quantification of nucleic acid target sequences with wide-ranging applications for both research and clinical diagnostic applications. ddPCR measures absolute quantities by counting nucleic acid molecules encapsulated in discrete, volumetrically defined water-in-oil droplet partitions. Droplet Digital PCR using Bio-Rad's QX100™ or QX200™ Droplet Digital PCR system overcomes the previous lack of scalable and practical technologies for digital PCR implementation.

ddPCR has the following benefits for nucleic acid quantification:

- Unparalleled precision — the massive sample partitioning afforded by ddPCR enables small fold differences in target DNA sequence between samples to be reliably measured
- Increased signal-to-noise — enrich for rare targets by reducing competition that comes from high-copy templates
- Removal of PCR efficiency bias — error rates are reduced by removing the amplification efficiency reliance of PCR, enabling accurate quantification of targets
- Simplified quantification — a standard curve is not required for absolute quantification

QX100/QX200 Workflow

Bio-Rad's QX100 or QX200 ddPCR system (Figure 1.1) combines water-oil emulsion droplet technology with microfluidics. The QX200 droplet generator partitions samples into 20,000 droplets (Figure 1.2). PCR amplification is carried out within each droplet using a thermal cycler. After PCR, droplets are streamed in single file on a QX200 droplet reader, which counts the fluorescent positive and negative droplets to calculate target DNA concentration.

Fig. 1.1. QX200 ddPCR system with associated consumables.

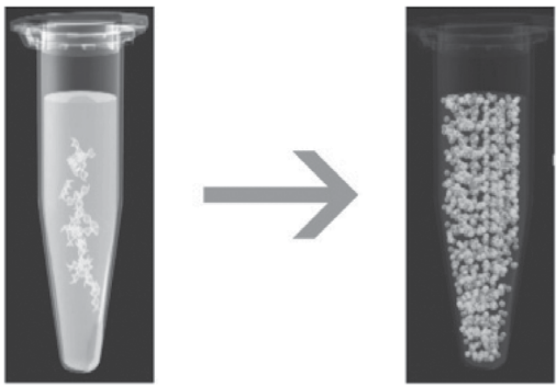


Fig. 1.2. In ddPCR, a single PCR sample is partitioned into 20,000 discrete droplets.

Droplet Generation

Before droplet generation, ddPCR reactions are prepared in a similar manner as real-time PCR reactions that use TaqMan hydrolysis probes labeled with FAM and HEX (or VIC) reporter fluorophores, or an intercalating dye such as EvaGreen.

ddPCR must be performed with the proprietary reagents developed specifically for droplet generation by Bio-Rad. Reagent mixes include the ddPCR supermix for probes and QX200 ddPCR EvaGreen supermix to partition DNA, and the one-step RT-ddPCR kit for probes to partition RNA.

Samples are placed into a QX100 or QX200 droplet generator, which uses specially developed reagents and microfluidics to partition each sample into 20,000 nanoliter-sized droplets. As shown in Figure 1.3, target and background DNA are distributed randomly into the droplets during the partitioning process.

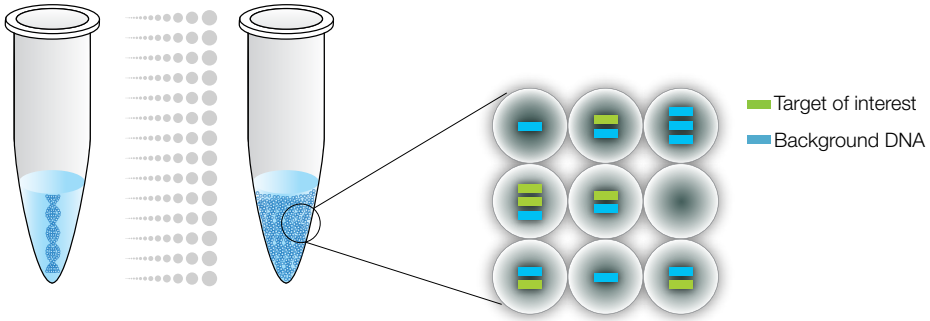


Fig. 1.3. In ddPCR, a single PCR sample is partitioned into 20,000 droplets.

Droplet generation produces uniform droplets for the sample, enabling precise target quantification (Figure 1.4).

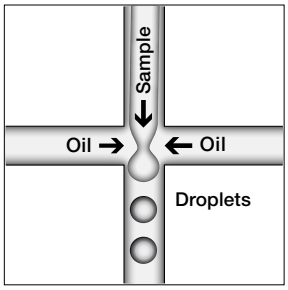


Fig. 1.4. The droplets created by the QX200 droplet generator are uniform in size and volume.

PCR Amplification

Droplets are transferred to a 96-well plate for PCR in a thermal cycler. We recommend the C1000 Touch™ thermal cycler with 96–deep well reaction module for PCR (Figure 1.5). This high-performance thermal cycler has excellent temperature uniformity and settling across all 96 wells to help ensure successful PCR.

Fig. 1.5. The C1000 Touch thermal cycler provides robust performance for ddPCR experiments.

Droplet Reading

Following PCR amplification of the nucleic acid target in the droplets, the plate containing the droplets is placed in a QX100 or QX200 droplet reader, which analyzes each droplet individually using a two-color detection system (set to detect FAM and HEX [or VIC]).

The autosampler of the droplet reader picks up the droplets from each well of the PCR plate (Figure 1.6).

Fig. 1.6. QX200 droplet reader autosampler.

Droplets are spaced out individually for fluorescence reading by the droplet reader (Figure 1.7). Fluorescence in two channels is then measured for individual droplets.

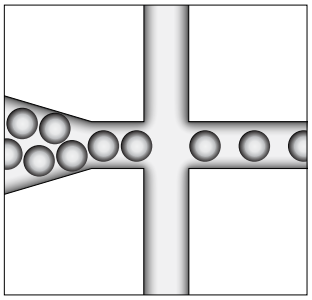


Fig. 1.7. Separating individual droplets in the QX100 droplet reader.

Positive droplets, which contain at least one copy of the target DNA molecule, exhibit increased fluorescence compared to negative droplets (Figure 1.8).

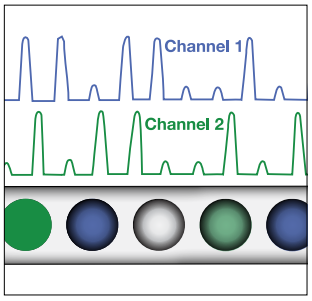


Fig. 1.8. Fluorescence readings are measured for each droplet in two channels.

ddPCR Data Analysis

Droplet Digital PCR data can be viewed as a 1-D plot with each droplet from a sample plotted on the graph of fluorescence intensity vs. droplet number. In Figure 1.9, all positive droplets, those above the red threshold line, are scored as positive and each is assigned a value of 1. All negative droplets, those below the red threshold line, are scored as negative and each is assigned a value of 0.

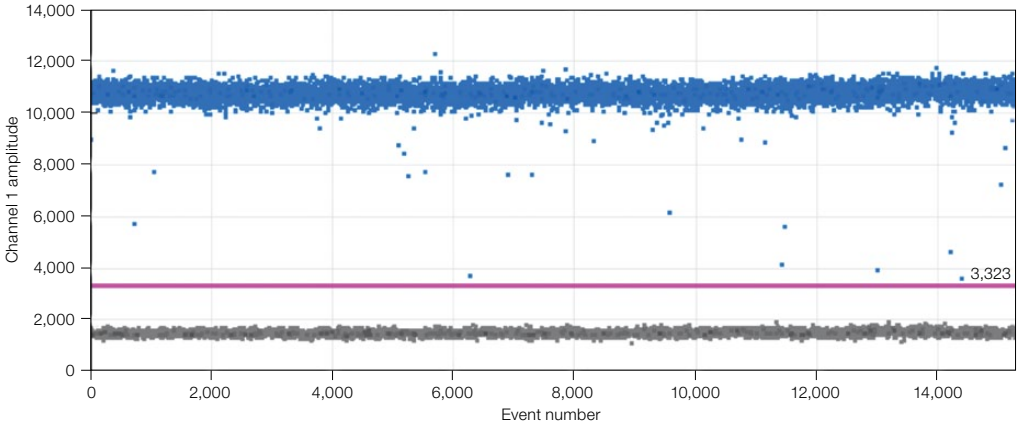


Fig. 1.9. Each droplet from a sample is plotted on the graph of fluorescence intensity vs. droplet number.

Droplet Digital PCR data from a duplex experiment in which two targets are PCR amplified can also be viewed in a 2-D plot in which channel 1 fluorescence (FAM) is plotted against channel 2 fluorescence (HEX or VIC) for each droplet (Figure 1.10).

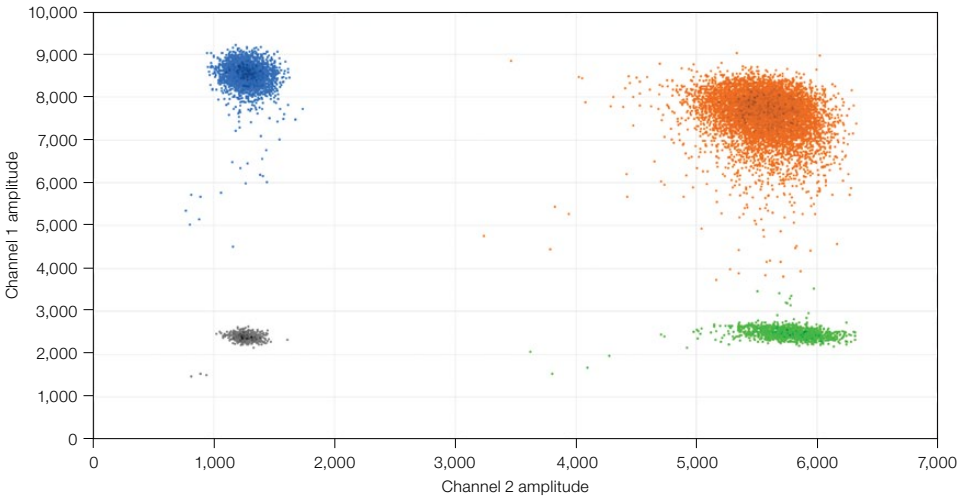


Fig. 1.10. 2-D plot of droplet fluorescence.

Because the DNA distribution into the droplets follows a random pattern, droplets cluster into four groups:

- FAM negative, HEX negative (double-negative droplets)
- FAM positive, HEX negative
- FAM negative, HEX positive
- FAM positive, HEX positive (double-positive droplets)

QuantaSoft™ software measures the number of positive and negative droplets for each fluorophore in each sample. The software then fits the fraction of positive droplets to a Poisson algorithm to determine the starting concentration of the target DNA molecule in units of copies/µl input (Figure 1.11).

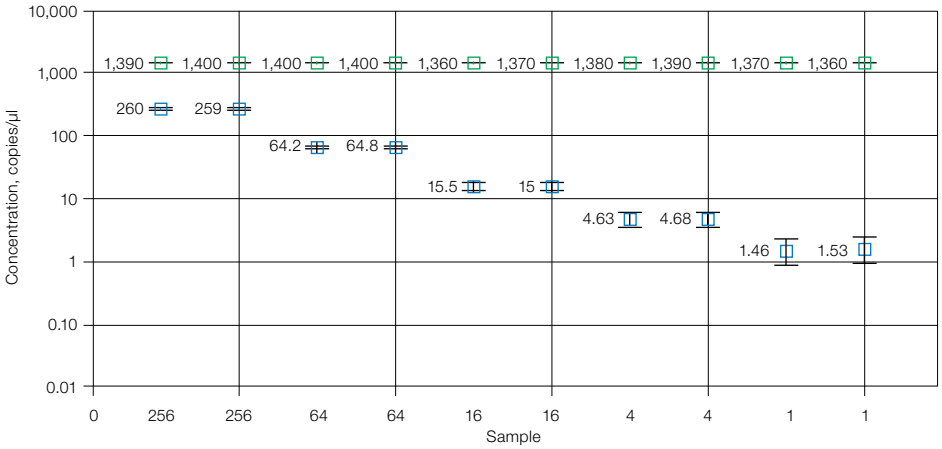


Fig. 1.11. Sample concentrations are plotted as copies/µl from the sample.

Emerging Applications of Droplet Digital PCR

Sample partitioning allows the sensitive, specific detection of single template molecules as well as precise quantification. It also mitigates the effects of target competition, making PCR amplification less sensitive to inhibition and greatly improving the discriminatory capacity of assays that differ by only a single nucleotide. Digital PCR offers the benefits of absolute quantification and greatly enhanced sensitivity. Therefore, its application in the following areas is growing:

- Absolute quantification — ddPCR provides a concentration of target DNA copies per input sample without the need for running standard curves, making this technique ideal for target DNA measurements, viral load analysis, and microbial quantification
- Genomic alterations such as gene copy number variation (CNV) — CNVs result in too few or too many dosage-sensitive genes responsible for phenotypic variability, complex behavioral traits, and disease. ddPCR enables measurement of 1.2x differences in gene copy number
- Detection of rare sequences — researchers must amplify single genes in a complex sample, such as a few tumor cells in a wild-type background. ddPCR is sensitive enough to detect rare mutations or sequences
- Gene expression and microRNA analysis — ddPCR provides stand-alone absolute quantification of expression levels, especially low-abundance microRNAs, with sensitivity and precision
- Next-generation sequencing (NGS) — ddPCR quantifies NGS sample library preparations to increase sequencing accuracy and reduce run repeats. Validate sequencing results such as single nucleotide polymorphisms or copy number variations with absolute quantification
- Single cell analysis — the high degree (10- to 100-fold) of cell-cell variation in gene expression and genomic content among homogeneous post-mitotic, progenitor, and stem cell populations drives a need for analysis from single cells. ddPCR enables low copy number quantification

ddPCR for Absolute Quantification and Experimental Considerations

In a typical digital PCR experiment, the sample is randomly distributed into discrete partitions such that some contain no nucleic acid template and others contain one or more template copies. The partitions are PCR amplified to end point and then read using a droplet reader to determine the fraction of positive partitions, from which the concentration is estimated by modeling as a Poisson distribution. The formula used for Poisson modeling is:

Copies per droplet = $-\ln(1 - p)$

where p = fraction of positive droplets.

Droplets are assigned as positive or negative by thresholding based on their fluorescence amplitude. The number of positive and negative droplets is used to calculate the concentration of the target and reference DNA sequences and their Poisson-based 95% confidence intervals (Figure 1.12).

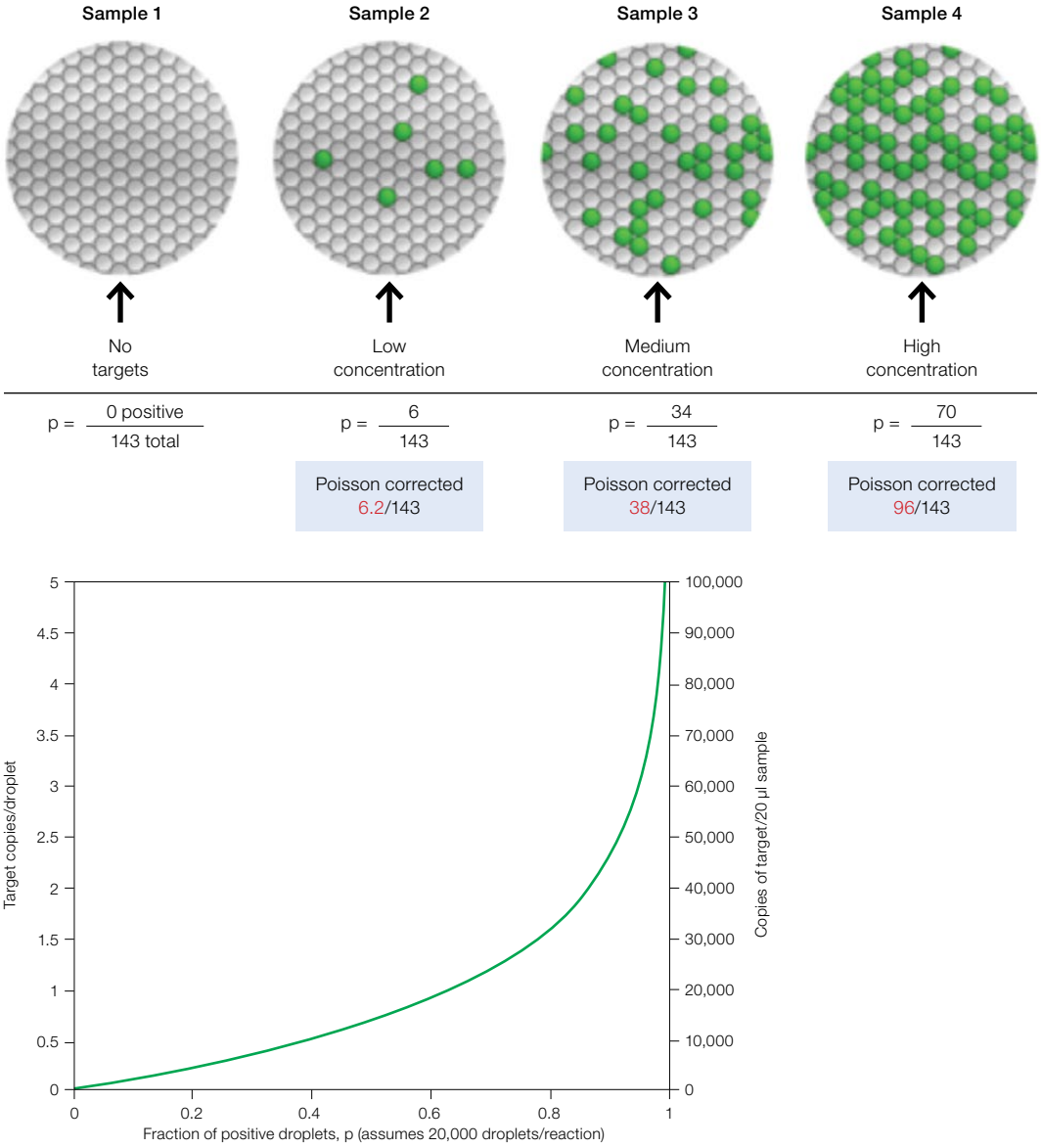


Fig. 1.12. Estimating target concentration by ddPCR.

ddPCR is an end-point measurement that enables you to quantify nucleic acids without the use of standard curves and independent of reaction efficiency. The present-or-absent digital format (Figure 1.12) and ability to draw clear thresholds between positive and negative droplet clusters for ddPCR means we now operate in a regime of absolute DNA quantification without the need for external calibrators or endogenous controls. The region separating these clusters is clear, such that the concentration output is not significantly affected by the position of the threshold and results are not biased by the small fraction of droplets that do not reach end point. This is another advantage of conducting many thousand PCR reactions/20 µl sample (Figure 1.13).

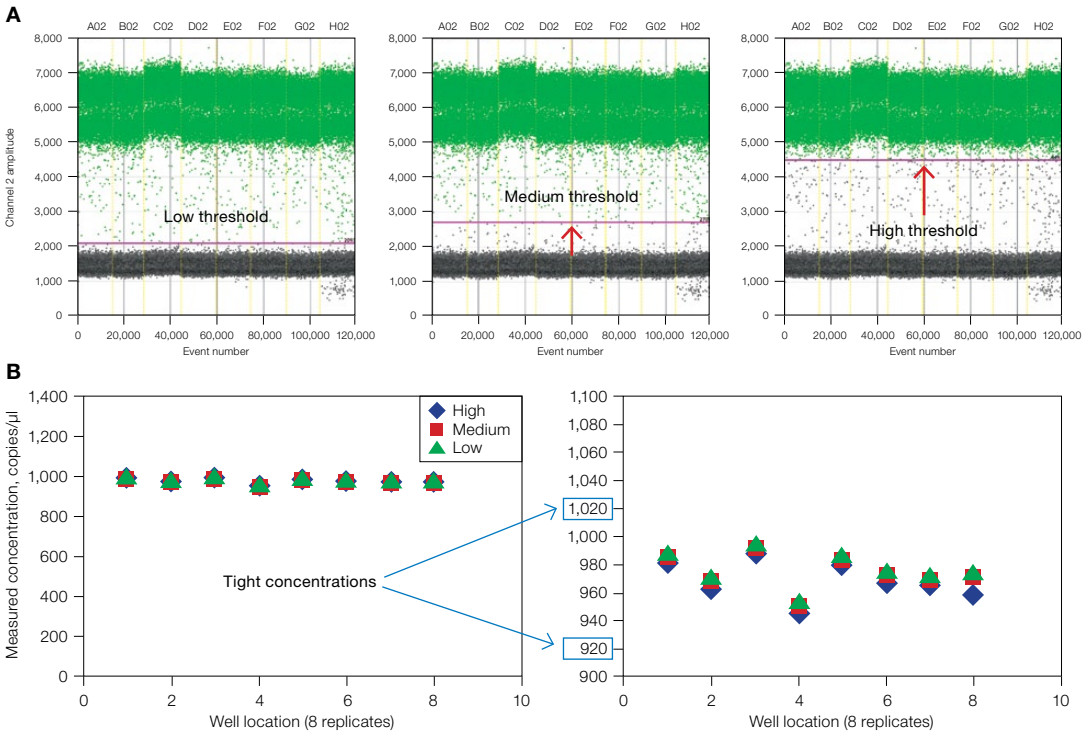


Fig. 1.13. The power of thousands of replicates using ddPCR. A, threshold options; **B**, measured concentration for different threshold settings.

Figure 1.13 shows the effect of varying the threshold value (Figure 1.13A) on concentration (Figure 1.13B). Eight ddPCR replicate wells are shown in the plots in Figure 1.13A. Three scenarios are tested for discriminating positive (high amplitude) from negative (low amplitude) droplets and the concentration calls associated with each (Figure 1.13B). A precision of ±5% is maintained regardless of the thresholding method used (see Fig. 1.13B zoomed-in view).

After optimizing the conditions to conduct a ddPCR experiment, the precision and resolving power afforded by partitioning are unprecedented. Figure 1.14A provides an example of this, showing a twofold serial dilution spanning 4 orders of magnitude of *Staphylococcus aureus* template concentration (■, FAM channel) in a constant background of human genomic DNA (gDNA) (■, VIC channel) run as a duplex assay. Figure 1.14B shows a zoomed-in view that displays the resolution of small copy number differences using ddPCR for a series of 10% dilutions of the same target and reference assays. The error bars represent Poisson 95% confidence intervals.

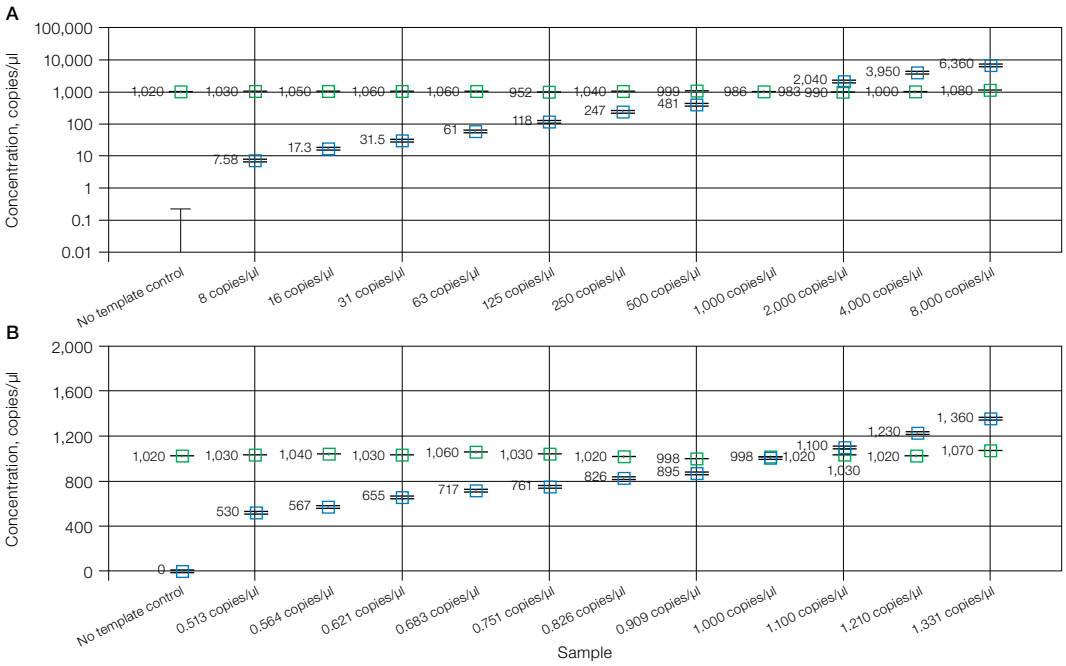


Fig 1.14. QuantaSoft software concentration estimates. Twofold (**A**) and 10% (**B**) dilution series of *S. aureus* template (■) in a constant background of human gDNA (■) run as a duplex FAM/VIC assay. The error bars represent Poisson 95% confidence intervals.

2 Designing Droplet Digital™ PCR Experiments

Assay Design for Droplet Digital PCR

As with any PCR-based technology, assay design and sample preparation are important for obtaining high-quality data. Before running a Droplet Digital (ddPCR™) experiment, know the goal or possible expected outcomes of the experiment because different types of experiments require different controls, sample preparation, amounts of DNA or RNA, and data analysis.

The amplification reaction of target molecules in ddPCR workflows follows similar principles of real-time PCR.

- Plan to amplify a 60–200 bp product
- Avoid regions that have secondary structure when possible
- Choose a region that, ideally, has a GC content of 40–60%

Designing Primers

Widely accepted quantitative PCR (qPCR) design guidelines apply to ddPCR primer design. Important criteria for single primers include melting temperature (T_m), length, base composition, and GC content. In addition, because primers are used in pairs, ensure that paired primers do not exhibit significant complementarity between 3' ends because this can result in primer-dimers. Extensive primer-dimer formations can significantly decrease or prevent amplification. The QX200™ Droplet Digital PCR system will support both hydrolysis probe (TaqMan) and DNA binding dye (EvaGreen) assays. All information in this chapter applies to both types of assay, except for the Designing Probes section.

When designing primers for a target sequence, follow these guidelines:

- Design primers that have a GC content of 50–60%
- Strive for a T_m between 50 and 65°C. One way to calculate T_m values is by using the nearest-neighbor method. Use the T_m calculator at **http://www.basic.northwestern.edu/biotools/oligocalc.html**, with values of 50 mM for salt concentration and 300 nM for oligonucleotide concentration
- Avoid secondary structure and adjust primer locations so they are outside the target sequence secondary structure, if required
- Avoid repeats of Gs or Cs longer than 3 bases
- Place Gs and Cs at the 3' nucleotide of primers when possible
- Check forward and reverse primer sequences to ensure no 3' complementarity (avoid primer-dimers)

Designing Probes

The QX100™ Droplet Digital PCR system is compatible only with TaqMan hydrolysis probes. The QX200 system is compatible with TaqMan hydrolysis probes and EvaGreen double-stranded DNA (dsDNA) binding dye. Using EvaGreen or SYBR® Green on the QX100 will damage the system.

Neither the QX100 nor the QX200 system is compatible with SYBR® Green. Advantages of using hydrolysis probes include high specificity, a high signal-to-noise ratio, and the ability to perform multiplex reactions. Hydrolysis assays include a sequence-specific, fluorescently labeled oligonucleotide probe in addition to the sequence-specific primers. TaqMan assays exploit the 5' exonuclease activity of certain thermostable polymerases. The hydrolysis probe is labeled with a fluorescent reporter at the 5' end and a quencher at the 3' end.

When the probe is intact, the fluorescence of the reporter is quenched due to its proximity to the quencher (Figure 2.1). The amplification reaction includes a combined annealing/extension step during which the probe hybridizes to the target and the dsDNA-specific 5' to 3' exonuclease activity of Taq or Tth cleaves off the reporter. As a result, the reporter is separated from the quencher, resulting in a fluorescence signal that is proportional to the amount of amplified product in the sample.

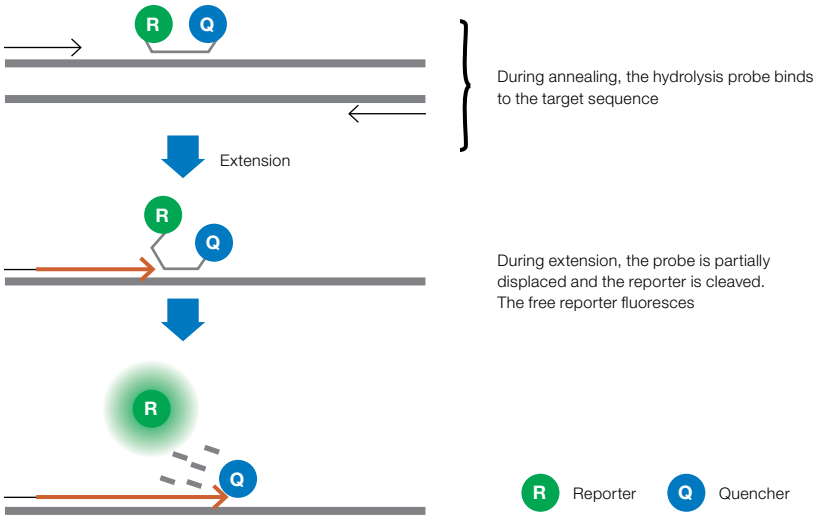


Fig. 2.1. In TaqMan assays, hydrolysis probes are hydrolyzed by Taq polymerase.

When designing probes, use the following guidelines:

- The probe sequence must be chosen between the two primers of the amplicon. Primer sequences cannot overlap with the probe, though they can sit directly next to one another
- The T_m of a hydrolysis probe should be 3–10°C higher than that of the primers
- Choose a sequence within the target that has a GC content of 30–80%, and design the probe to anneal to the strand that has more Gs than Cs (so the probe contains more Cs than Gs)
- The probe should be <30 nucleotides long because distance between fluorophore and quencher affects baseline signal intensity. Longer probes or T_m enhancers are recommended if needed to achieve the required T_m
- Probes should not have a G at the 5' end because this quenches the fluorescence signal even after hydrolysis
- Black Hole Quencher or other nonfluorescent quenchers are recommended
- T_m enhancers for probes are recommended for single nucleotide polymorphism (SNP) and rare mutation detection assays in order to keep the background fluorescence to a minimum. Shorter probes discriminate better between single base differences in the target amplicon(s)

The QX100 and QX200 systems are compatible with FAM and HEX or VIC dyes as well as duplex analysis of FAM/HEX or FAM/VIC. Using dye combinations in duplex assays enables a multiplex experiment and the ability to quantify and/or detect up to two targets per sample.

Designing an Assay

Because manual balancing of all the constraints for primer and probe designs is challenging, time consuming, and error prone, we recommend software tools to ensure that all design constraints are satisfactorily followed. One widely used tool for PCR assay design is Primer3 (Whitehead Institute for Biomedical Research, Massachusetts Institute of Technology [MIT]). Building on this software, the easy-to-use Primer3Plus web interface (Untergasser et al. 2007) makes the process of assay design even easier (Figure 2.2).

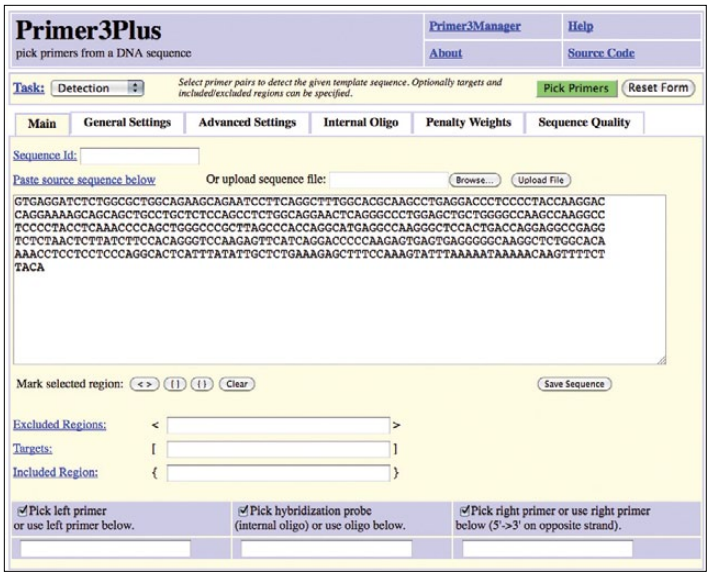


Fig. 2.2. Main tab in Primer3Plus.

In the Main window, paste your target DNA sequence in the “Paste source sequence below” field.

We recommend the following changes to the default settings when designing ddPCR assays:

- In the General Settings window, change “Concentration of divalent cations” to 3.8, “Concentration of dNTPs” to 0.8, and “Mispriming/Repeat Library” to the correct organism
- In the Advanced Settings window, change both the “Table of thermodynamic parameters” and “Salt correction formula” to SantaLucia 1998
- In the Internal Oligo window, we recommend setting 15 for the minimum number of bases for the oligo. We recommend 64°C as the minimum T_m for the probe, 65°C as the optimal T_m for the probe, and 70°C as the maximum T_m for the probe. These parameters can be relaxed to allow for smaller/larger oligos, which may be necessary for high GC or low GC targets. Oligo size should be no smaller than 13 and no larger than 30 nucleotides

Note: After you have made the desired changes in Primer3Plus, select **Save Settings** under General Settings and save these parameters in a file. To apply these settings in the future, upload them by selecting **Browse** in the General Settings tab, find this file, and click **Activate Settings**.

After you paste your target sequence into the Main window, click **Pick Primers** (Figure 2.3). The software provides one or more primer pairs to select or provides an explanation for why the software failed to arrive at any primers.

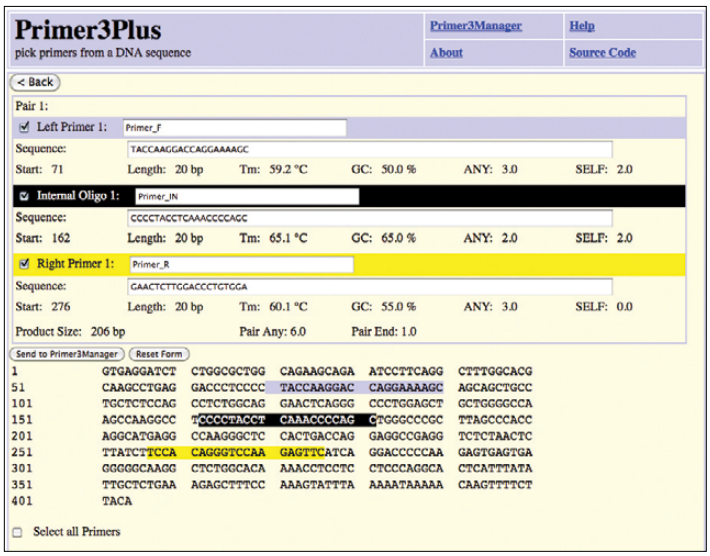


Fig. 2.3. Assay design output.

Several important design features are not addressed by Primer3Plus (or the Primer3 MIT site).

- To ensure primer specificity, use tools such as BLAST (Basic Local Alignment Search Tool), hosted at the National Center for Biotechnology Information (NCBI), either in the "traditional" general search form (**www.ncbi.nlm.nih.gov/BLAST**) or a form tailored specifically to check that PCR primers (**http://www.ncbi.nlm.nih.gov/tools/primer-blast/**) match only your intended target
- Check that common SNPs do not land in your primer sequences
- Check for the secondary structure of the amplicon using the Mfold program (**http://mfold.rna.albany.edu/?q=mfold**). Primer binding sites should be predicted to be "open" (that is, not base-paired in a secondary structure) at the PCR annealing temperature

Sample Preparation

The quality of the nucleic acid preparation from the sample of interest can impact ddPCR results. An optimized protocol should be used to extract the DNA or RNA from the raw material you are testing. Ensure that the sample has not been degraded, for example, by heating above 60°C. Although some PCR inhibitors are less detrimental to quantification accuracy in ddPCR than in other technologies, we recommend removing as many of these as possible during the nucleic acid purification phase. If known inhibitors cannot be readily removed, consider reducing their impact on the PCR reaction by diluting the sample 1:10.

For best results, restriction digestion of your DNA sample outside of the amplicon region is recommended. We have extensively investigated the use of endonucleases for fragmentation and found that a wide range of enzymes with 4-base and 6-base recognition sites perform satisfactorily for this purpose. The benefits of predigestion can be achieved with a wide range of enzyme concentrations. Considerations should be taken into account in the choice of enzyme for a particular locus:

1. The enzyme should not cut within the PCR target sequence itself.
2. It is best to use an enzyme that is insensitive to methylation to avoid incomplete fragmentation due to methylation of the target DNA.
3. In some instances, it is best to digest the target copy to the smallest size fragment that fully contains the amplicon footprint — preferably less than a few hundred base pairs.
4. If added at a relatively high concentration, some restriction enzyme buffers can result in a significant change in salt concentrations of the reaction mix. To avoid this, always digest in the lowest possible volume and mix with water before adding the digested DNA to the reaction mix.

Adding DNA to the Reaction Mix

The recommended dynamic range of the QX100 system is from 1 to 120,000 copies/20 µl reaction. There are about 120,000 copies in 400 ng of human DNA, assuming 1 copy/haploid genome. To estimate the number of copies/ng of DNA for your organism you must know the mass or the number of base pairs in the genome (see formula below). If the experiment entails quantifying samples known to have extremely high amounts of target molecules (such as next-generation sequencing [NGS] libraries), plan to reduce the starting sample accordingly. If the target copy number/genome is unknown, we recommend that you determine the optimal starting amount by doing four tenfold dilution series of each sample at the expected digital range. By assaying the four data points above and below the expected digital range, you ensure that one of the data points is within the optimal digital range.

To help determine copy number per genome, collect the following information:

1. If the source or species of the gDNA is known but the genome size of the organism of interest is unknown, refer to **http://www.cbs.dtu.dk/databases/DOGS/index.html** to determine the size of the genome in question.
2. Once the size of the genome is known, determine the mass of the genome using the following formula:

   $m = (n)\ (1.096 \times 10^{-21}\ \text{g/bp})$

   where m is the genome mass in grams, and n is the genome size in base pairs.

The following example calculates the mass of the human genome using the Celera Genomics estimate of

3.0×10^{9} bp (haploid):

$m = (3.0 \times 10^{9}\ \text{bp})\ (1.096 \times 10^{-21}\ \text{g/bp})$

$m = 3.3 \times 10^{12}$ g or 3.3 pg

The example is relevant to any gene that is present at the normal rate of 2 copies/diploid genome, such as *RPP30*, and provides a basis for a digital screening experiment to determine the optimal digital range.

For sample DNA loading, follow these guidelines:

- Assess input DNA/RNA concentration using A_{260} spectroscopy to ensure the target DNA/RNA concentration is being loaded within the dynamic range of detection

Note: An A_{260} measurement depends on various factors and does not distinguish between intact and fragmented targets. As such it provides only an estimate of your DNA concentration.

- Add no more than 1 µg of digested DNA to the 20 µl reaction (final concentration of 50 ng/µl)
- Intact DNA requires restriction digestion for optimal performance, especially at a concentration above 3 ng/µl (60 ng/20 µl reaction). For copy number and absolute quantification, always do restriction digestion at all DNA concentrations unless you want to access proximal replicate sequences
- Do not perform a restriction digestion of the DNA sample within the amplicon sequence
- Fragmented DNA (for example, formalin-fixed, paraffin-embedded [FFPE] samples) may not need restriction digestion. However, some assays and/or targets require digestion for optimal target detection regardless of fragmentation
- cDNA does not require restriction digestion

ddPCR Experimental Workflow

Droplet Generation

For droplet generation, transfer 20 µl of a PCR reaction containing sample nucleic acid, primers (and probes for a TaqMan experiment), and the appropriate Bio-Rad ddPCR supermix to the middle rows of a DG8™ cartridge for a QX100 or QX200 droplet generator (Figure 2.4). We recommend creating an initial reaction pool that is slightly more than 20 µl (22–25 µl) to ensure that 20 µl of mixture is transferred to the DG8 cartridge. Reaction mixes should be combined and well mixed in a separate tube and not in the droplet generator cartridge. Reaction mixes should then be transferred to the DG8 cartridge already preloaded in the DG8 cartridge holder.

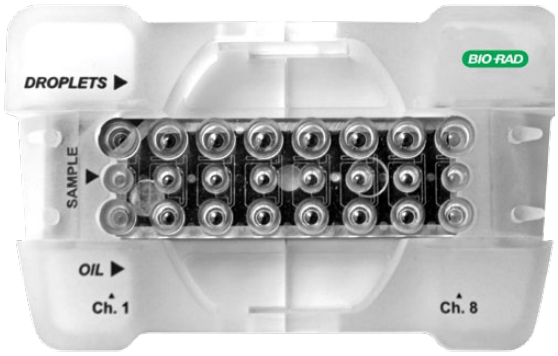


Fig. 2.4. Loaded DG8 cartridge.

Note: Each DG8 cartridge generates eight wells of droplets. Any unused wells on the cartridge must be filled with 1x ddPCR buffer control.

The Bio-Rad ddPCR supermixes have been formulated specifically to work with the droplet chemistry. Altering the components used in the QX100 droplet generator or using a different supermix will negatively impact results. A 1x final concentration of supermix must be used for proper droplet formation and proper target quantification.

After loading a 20 µl PCR reaction, load 70 µl of droplet generation oil into the bottom wells of the DG8 cartridge (Figure 2.4). Attach a gasket across the top of the DG8 cartridge and place it into the QX200 droplet generator (Figure 2.5). The droplet generator produces about 20,000 droplets per sample in about 2.5 min for eight samples. Droplets should be transferred to a 96-well PCR plate by pipetting gently.

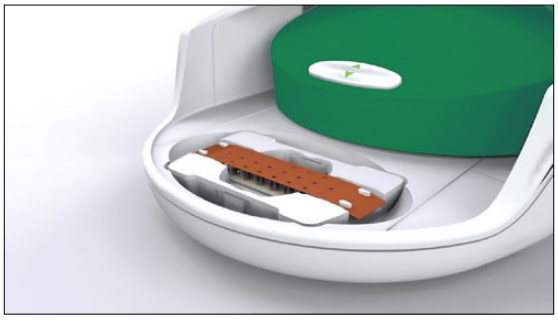


Fig. 2.5. Loaded DG8 cartridge placed in the QX200 droplet generator.

We recommend designing your experimental plate layout on a 96-well plate in columns because the cartridge contains eight wells.

PCR

After generating droplets in the DG8 cartridge, pipet the droplets from the top wells of the cartridge into a PCR plate. The PCR plate should be heat sealed using Bio-Rad's PX1™ PCR plate sealer and pierceable foil heat seal.

Note: Using an alternative seal with glue can damage the droplet reader.

After heat sealing, place the PCR plate in a thermal cycler for PCR using the following guidelines.

- Use a recommended thermal cycling protocol
- Use a 2.5°C/sec ramp rate to ensure each droplet reaches the correct temperature for each step during the cycling
- 40 cycles of PCR is enough for an optimized ddPCR assay. Do not exceed 50 cycles
- After PCR, the plate can be left in the thermal cycler overnight at 10°C or stored at 4°C. Do not store the plate for more than 3–4 days before running it in a QX100 or QX200 droplet reader

Setting Up an Experiment in QuantaSoft™ Software

From the computer attached to the droplet reader, open QuantaSoft software in the setup mode and design a new plate with a layout according to your experimental design. Detailed instructions for how to set up a new experiment and interpret ddPCR data can be found in the user manual.

Double click on a well in the plate layout to open the Well Editor dialog box. Designate the sample name, experiment type, and which assays correspond to which channels, such as FAM and HEX (Figure 2.6). You can select several contiguous wells at one time using shift + double click or select non-contiguous wells using Ctrl + double click. Either selection will bring up the labeling menu. In the Well Editor dialog box, input sample names and use the dropdown menu to designate the experiment type.

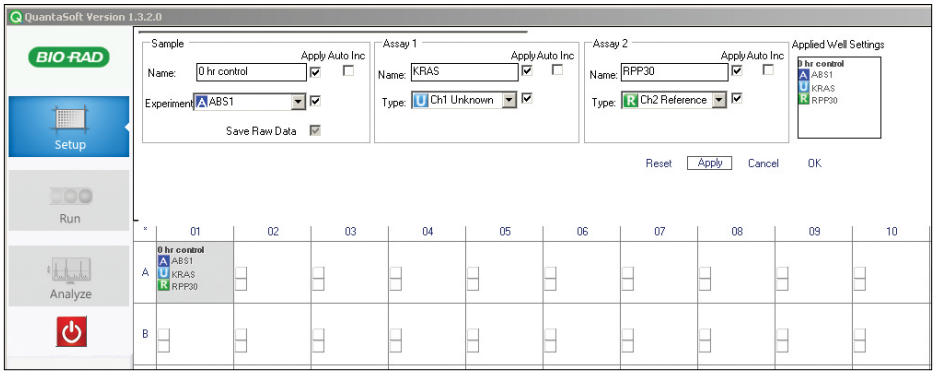


Fig. 2.6. Labeling the plate.

There are three types of experiments that can be selected for each well:

- ABS — absolute quantification
- RED — rare target sequence detection (rare event detection)
- CNV — copy number variation to measure the concentration of target relative to the concentration of a reference

Select **Apply** to load the wells and when finished select **OK**. Once the plate layout is complete, select **Run** to begin the droplet reading process.

Droplet Reading

Following PCR amplification of the nucleic acid target in the droplets, place the PCR plate in a QX100 or QX200 droplet reader. The droplet reader and QuantaSoft software count the PCR-positive and PCR-negative droplets to provide absolute quantification of target DNA.

Droplet reading considerations are as follows:

- Before a run, the instrument can be set to interrogate droplets either in rows or columns
- Ensure there is enough droplet reader oil in the instrument and the waste is empty before a run
- Each sample is processed individually and interrogated for both FAM and HEX (or VIC) fluorescence
- Data from 12,000–16,000 droplets are used in concentration calculations
- The reader measures fluorescence intensity of each droplet and detects the size and shape as droplets pass the detector; droplets are excluded if they do not meet quality metrics

Data Analysis

After the QX100 or QX200 droplet reader has finished interrogating all wells, use QuantaSoft software to analyze the data in each well. It automatically opens the first well with data to begin the analysis. If the plate was set up for ABS analysis, automatic thresholding determines concentrations and populates the data tables in the analysis mode of the software.

Important: The concentration reported is copies/µl of the final 1x ddPCR reaction.

Well data must meet certain quality metrics before QuantSoft software will automatically calculate a threshold above which droplets are considered positive. The threshold may be manually adjusted on a well-by-well basis or across an entire plate (Figure 2.7).

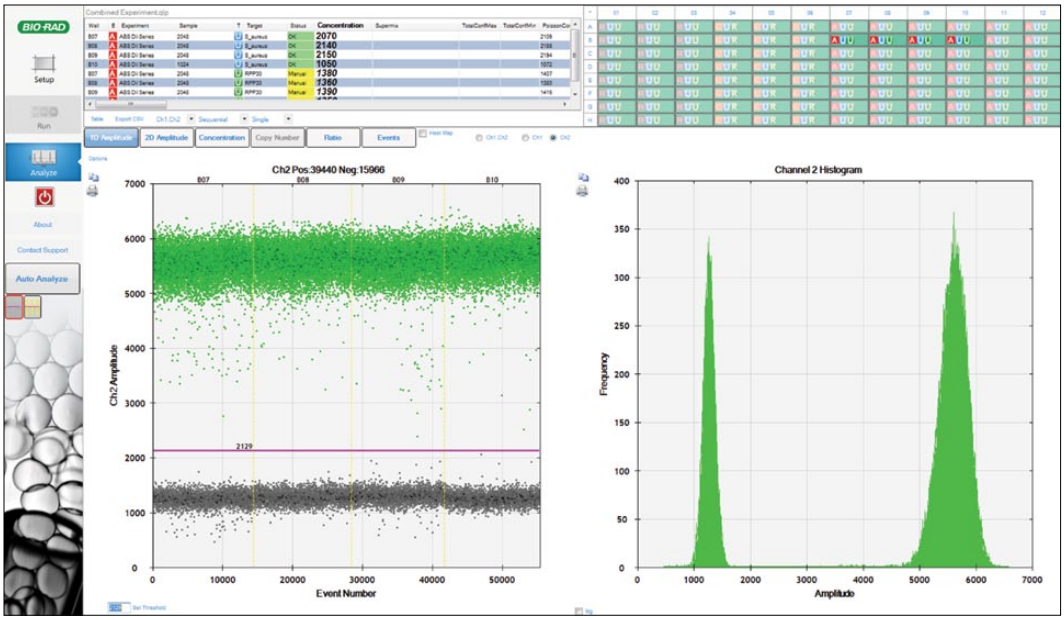


Fig. 2.7. Setting thresholds in a 1-D plot.

Note: Use 1-D plots for temperature gradient experiments and wells containing single assays. For all experiments involving duplex assays, set thresholds in the 2-D plot view (Figure 2.8).

For correct quantification of a single-color experiment, use the thresholding tools to ensure correct designation of the populations as negatives (gray) and positives (blue or green). The thresholding tools are on the left side of the screen. There is an autoanalysis tool as well as two manual thresholding tools — one for individual wells and one for all selected wells. Once you have chosen the desired tool, place the crosshair cursor over the place where you would like to set the threshold and click on the chart. The droplets will change color when you are done.

To analyze the data for a multiplex experiment, select **Analyze** and click the **2-D Amplitude** tab.

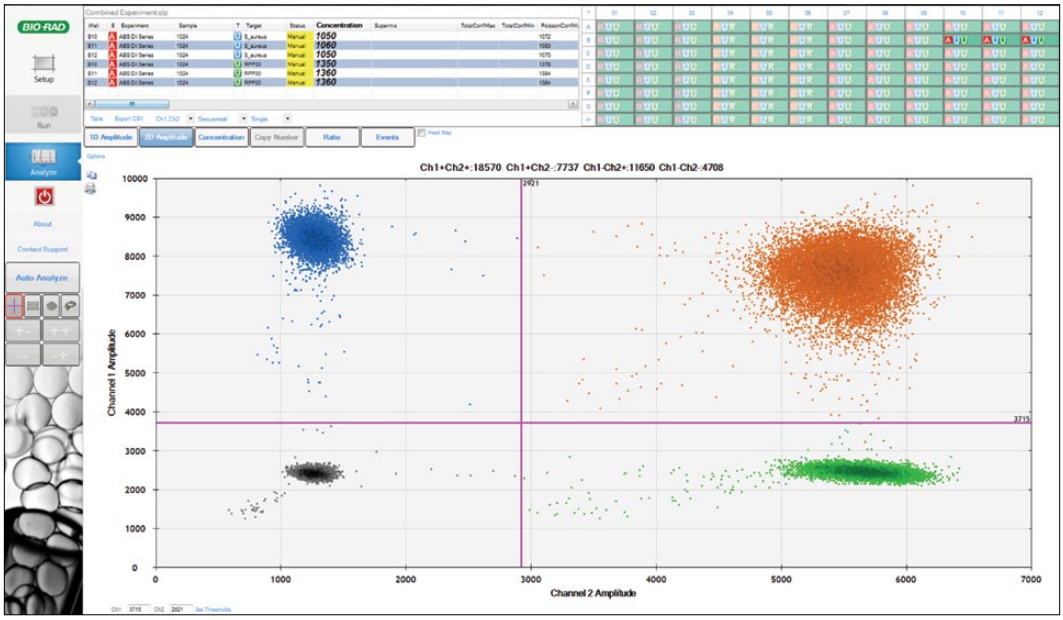


Fig. 2.8. Setting thresholds in a 2-D plot.

For correct quantification of a duplex experiment, use the thresholding tools to ensure correct designation of the droplet populations as double negative (gray), FAM positive (blue), VIC/HEX positive (green), and double positive (brown = positive for FAM and HEX/VIC in the same droplet). There is an autoanalysis tool as well as several different manual thresholding tools including a "free-draw" tool. Once you have selected the desired color tab and draw tool, click on the chart and select the droplets. They will change color when you are done.

Merging Wells

Selecting the merged button in QuantaSoft software combines the positive and negative droplets from all the wells with the same name and analyzes these data together as a single experiment. You can display merged and single wells simultaneously or individually. All merged-well data are displayed with a closed symbol (Figure 2.9). In contrast, single-well data are displayed with an open symbol (see Figure 1.11).

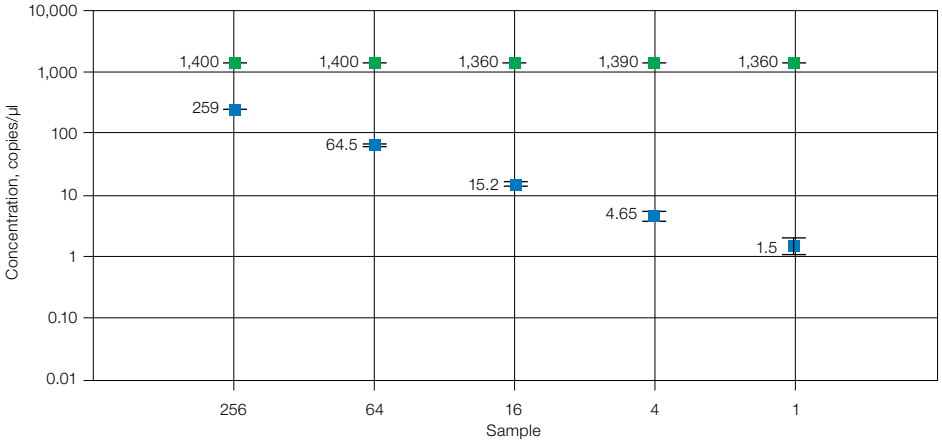


Fig. 2.9. Merged well data.

PCR Optimization Using Thermal Gradients

Optimizing the annealing temperature of your PCR assay is one of the most critical parameters for reaction specificity. Setting the annealing temperature too low may lead to amplification of nonspecific PCR products. On the other hand, setting the annealing temperature too high may reduce the yield of a desired PCR product. Even after calculating the T_m of a primer, you may need to determine the optimal annealing temperature empirically because this temperature depends on buffer conditions as well as primer and probe concentrations. This involves repeating a reaction at many different temperatures. Similar time-consuming tests may also be required to optimize the sample denaturation temperature.

As with classical PCR reactions, the optimal annealing temperature for an assay can be easily determined using thermal cyclers that have a thermal gradient feature. The C1000 Touch™ thermal cycler offers a gradient feature. The gradient feature enables you to test a range of temperatures simultaneously, optimizing the annealing temperature in a single experiment.

To find the optimal annealing temperature for your reaction, test a range of temperatures above and below the calculated T_m of the primers. The optimal annealing temperature is the one that results in the largest fluorescence amplitude difference between the positives and negatives and that avoids nonspecific amplification. It is also important to view the concentration values obtained across the thermal gradient simultaneously when considering the fluorescence amplitude separation of positives and negatives. A sample annealing temperature optimization experiment is shown in Figure 2.10 demonstrating separation of positives and negatives of replicate samples across the thermal gradient.

For two-color assays, a thermal gradient can be used to identify a temperature where both assays perform well.

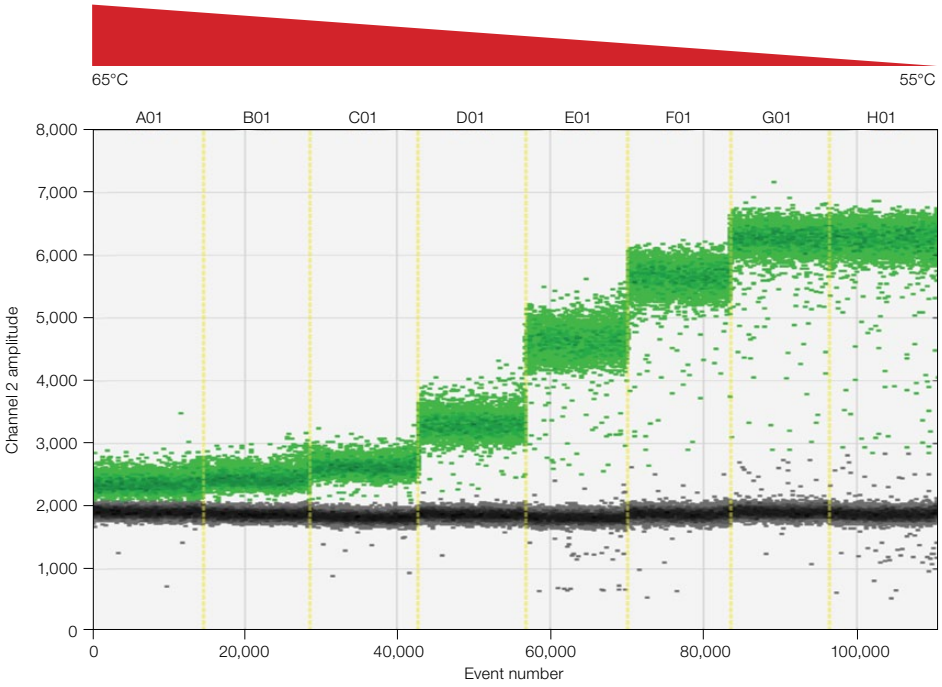


Fig. 2.10. Thermal gradient optimization.

ddPCR Using the QX200 System and EvaGreen dsDNA Dye

The QX200 system can measure amplified DNA using Bio-Rad's QX200 ddPCR EvaGreen supermix, template, and a pair of primers. EvaGreen dsDNA binding dye is similar to SYBR® Green in that it fluoresces upon binding double-stranded DNA (Figure 2.11).

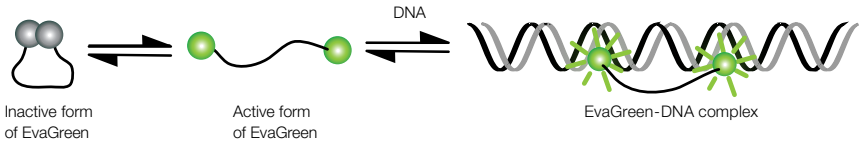


Fig. 2.11. EvaGreen dye binds to dsDNA via a "release-on-demand" mechanism.

EvaGreen dsDNA binding dye enables double-stranded DNA detection with the convenience and savings of only needing primers to amplify and detect product with the added high-resolution features of ddPCR. ddPCR with EvaGreen can be used with applications such as gene expression, copy number variation, DNA rearrangement detection, micro RNA detection, and multiplexing.

The fluorescence amplitude of each droplet with EvaGreen varies with amplicon length and with PCR efficiency. Longer amplicons will bind to more EvaGreen dye molecules and therefore be brighter. PCR reactions that are not at their most efficient annealing/extension temperature will result in fewer amplicons in each positive droplet at end point and therefore lower fluorescence amplitude. Despite the range in positive fluorescence amplitude, accurate quantification is still routinely achieved. These features provide the user the ability to measure length or PCR efficiency as well as the ability to measure multiple targets in the same well while only using primers. Additionally, primer-dimers and off-target amplicons can be detected as low fluorescence amplitude droplets. With the EvaGreen supermix reactions it is possible to simply set the threshold above the primer-dimer in order to obtain an accurate quantification of the target without having to redesign the primers (Figure 2.12).

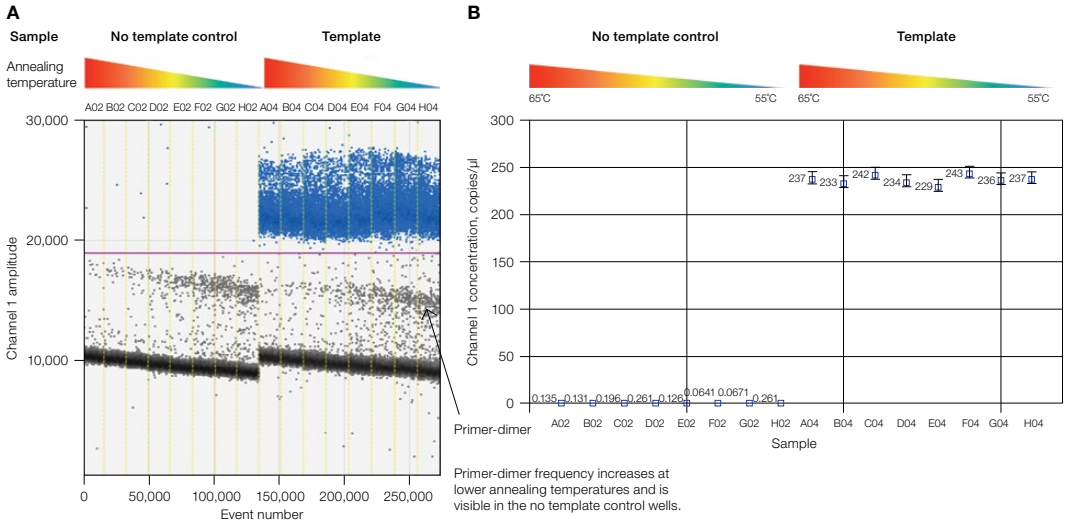


Fig. 2.12. Accurate quantification of target is obtained using Bio-Rad's QX200 system with the QX200 ddPCR EvaGreen supermix. A, threshold set above primer-dimer; **B**, accurate quantification of the target is obtained without redesigning primers.

In Figure 2.13, a gradient of annealing/extension temperatures is shown for three amplicons with different lengths: A, B, and C correlate to 200, 99, and 62 bp amplicons, respectively. The experiment demonstrates the effect amplicon length has on fluorescence amplitude as well as how annealing/extension temperature affects amplitude and the ability to resolve products with different lengths based on amplicon size. Red arrows show increasing separation between 200 and 99 bp amplicons as the annealing/extension temperature is lowered from 65 to 55˚C.

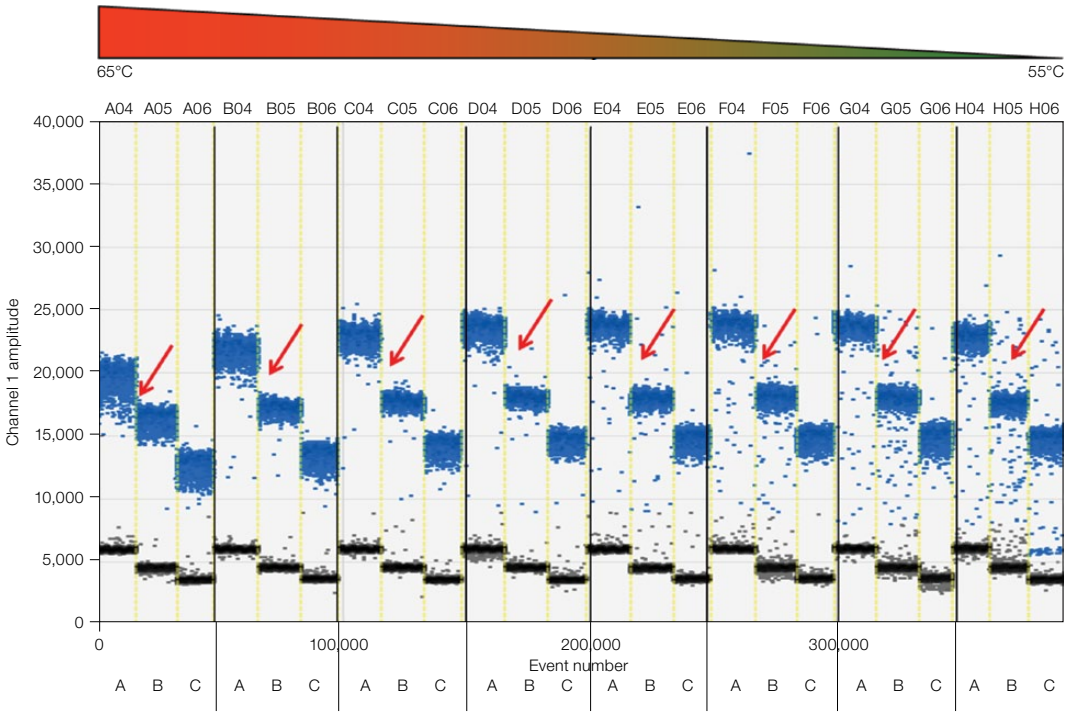


Fig. 2.13. Gradient of annealing/extension temperatures for three amplicons with different lengths: A (200 bp), B (99 bp), and C (62 bp).

EvaGreen and Gene Expression

Measuring gene expression using ddPCR and EvaGreen allows you to detect and quantify splice variants as well as contaminating genomic DNA. Figure 2.14 shows an experiment demonstrating that splice variants can be seen as a cluster of droplets with higher fluorescence amplitude, referred to as a super cluster. Control samples with no reverse transcriptase added were run to verify that the super cluster was not due to residual genomic DNA.

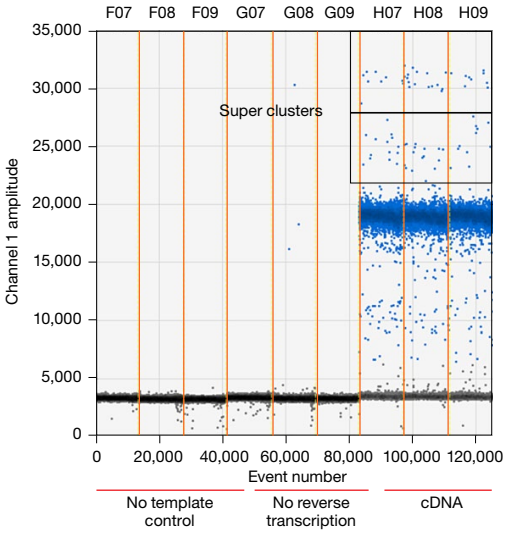


Fig. 2.14. Splice variant discrimination using the QX200 ddPCR EvaGreen supermix with a single set of primer pairs.

Multiplexing with EvaGreen

Differences in droplet amplitude due to differences in amplicon length or optimal annealing temperature allow for multiplexing in a single well. Figure 2.15 shows a 2-D fluorescence plot containing two amplicons, where differences in amplitude are observed due to differences in optimal annealing temperature. Primer set 1 has an optimal T_m of 63˚C, therefore droplets having this target have relatively high fluorescence amplitudes at 63˚C. Primer set 2 has an optimal annealing/extension temperature of 59˚C and therefore has a much lower fluorescence amplitude at 63˚C. Distinct separation of these clusters allows a copy number to be calculated with high precision (Figure 2.15). Similarly, varying amplicon length instead of PCR efficiency can be used to measure multiple targets in the same well. However, when using amplicon length to multiplex with EvaGreen it is important to use an optimal annealing/extension temperature for thermal cycling (Figure 2.13).

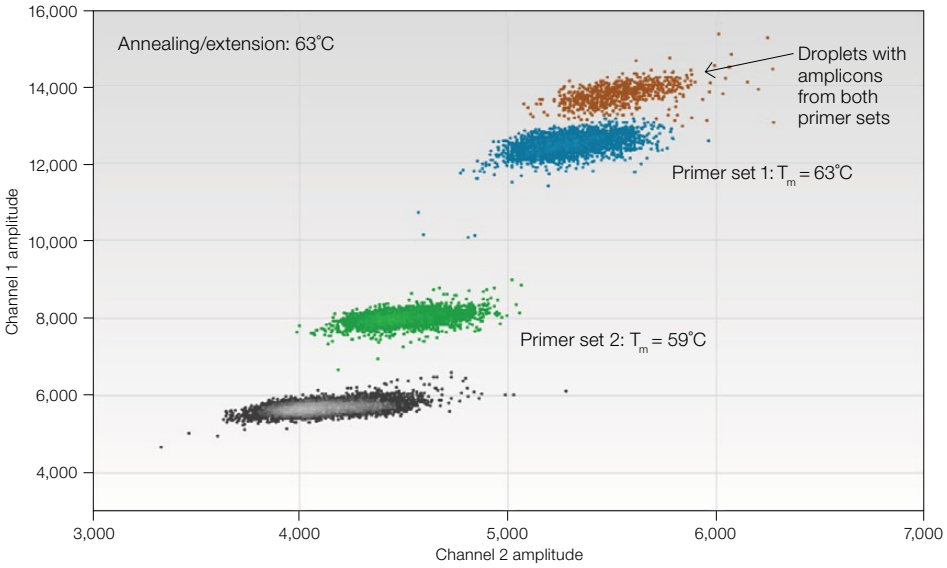


Fig. 2.15. Multiplex assays performed using ddPCR with EvaGreen. Differences in amplitude are due to differences in optimal annealing temperature. T_m, melting temperature.

Reference

Untergasser et al. (2007). Primer3Plus, an enhanced web interface to Primer3. Nucleic Acids Res 35 (web server issue), W71–W74.

3 Absolute Quantification and the Statistics of Droplet Digital™ PCR

Running Absolute Quantification Experiments

Absolute quantification (ABS) is fundamental to all Droplet Digital PCR (ddPCR™) applications. Partitioning template DNA into uniform droplets enables highly quantified measurements of target DNA using the QX200™ or QX100™ Droplet Digital PCR system and the appropriate automatic statistical analysis in QuantaSoft™ software. ddPCR does not require a standard curve, and this simplifies the experimental planning and procedures, and reduces result variability and cost.

Important ABS experiment considerations are as follows:

- Digest the template DNA to fragment the genome. Select restriction enzymes that do not cut either the target or reference amplicons
- The restriction-digested DNA should be at a higher concentration in order to have a lower concentration in the master mix

Note: Account for the concentration of the template in any restriction digest and the amount of template in the final 1x ddPCR supermix to backcalculate the original concentration of the stock template.

Note: Do not exceed 5,000 copies of target/µl of the final ddPCR reaction mix.

- Use a thermal gradient to optimize ddPCR results
- In the Well Editor of QuantaSoft software, designate ABS for the experiment type
- In the Well Editor, indicate which assay is the target and which assay is the reference when performing multiplex reactions

Absolute Quantification Data Analysis

The ABS experiment is designed to quantify the concentration of the target and give a result in copies/µl of the final 1x ddPCR reaction.

If the plate was set up for ABS analysis, automatic thresholding determines concentrations and populates the data tables in the analysis mode of the software. The threshold may be manually adjusted. Note specifically any wells that are flagged as No Call in the status column of the data tables.

QuantaSoft software will return a No Call for wells with too many positive droplets (not enough empty droplets to apply Poisson statistics), wells with Quality Scores below 0.85, and wells with fewer than 10,000 droplets. After visually inspecting the data, you may set a threshold manually and QuantaSoft software will complete the calculations.

Figure 3.1 shows excellent separation between positive droplets (green) and negative droplets (black) in the chart on the left, showing droplets (event number) vs. fluorescence amplitude. The histogram on the right plots amplitude vs. the frequency of the populations of droplets and can assist in setting the threshold.

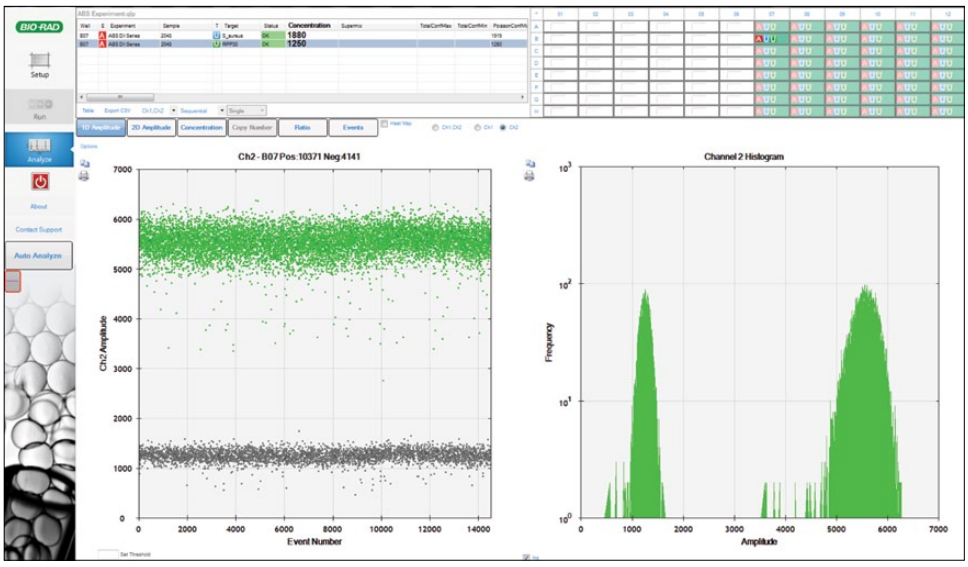


Fig. 3.1. Example of a well-performing assay for absolute quantification.

QuantaSoft software measures the number of positive and negative droplets for each fluorophore in each sample. It then fits the fraction of positive droplets to a Poisson algorithm to determine the starting concentration of the target DNA molecule in units of copies/µl input from the sample (Figure 3.2).

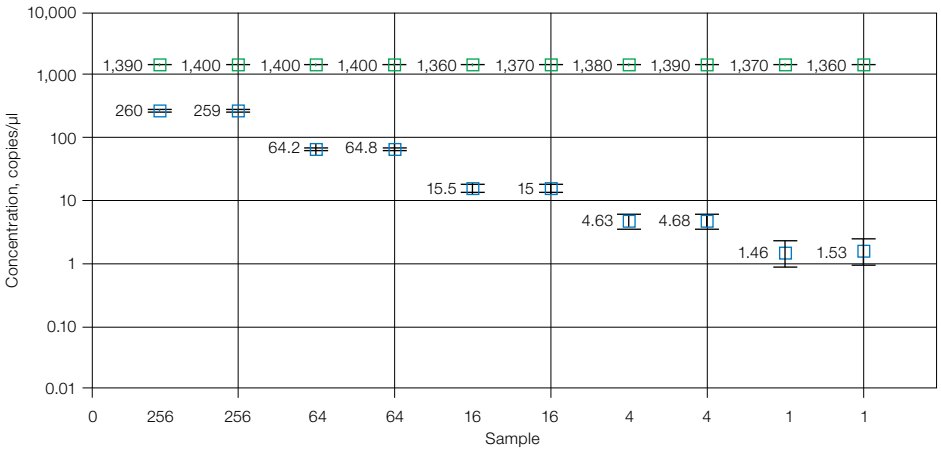


Fig. 3.2. Sample concentrations are plotted as copies/µl from the sample.

Statistics of ddPCR

In ddPCR, DNA molecules and PCR reagents are partitioned into droplets. Some droplets will contain one or more copies of the target DNA molecule (DNA fragment that contains primer and probe binding sites for a given assay) and some will not contain any copies of the target. Droplets are subjected to PCR amplification and the targeted PCR product, with an associated fluorescence signal, is generated in droplets that initially contained the target DNA. The concentration of the target species in the original sample is determined from the fraction of droplets that have a fluorescence signal.

In ddPCR, target molecules are distributed randomly into droplets. This assumption is at the heart of all digital PCR approaches. Random partitioning means a given target molecule is equally likely to end up in any of the 20,000 droplets. Random partitioning also means target molecules move independently of each other and do not interact with each other.

The droplet generator partitions samples into approximately 20,000 droplets of identical volume (about 1 nl). Some droplets are lost in transfer steps and others are eliminated by the stringent metrics applied by QuantaSoft software as the droplets pass through the droplet reader, resulting ultimately in data from 12,000–16,000 droplets being used in subsequent concentration calculations. Reading only a subset of the total droplets has no impact on the concentration measurement, because the concentration is calculated based on the fraction of droplets that is empty (that is, the fraction that does not contain any target DNA).

As an example, suppose a sample contains 1,000 copies/µl of a target molecule, and the sample is loaded into two ddPCR wells. In one well, only 10,000 droplets are read by the software, and in the other, 15,000 droplets are read. In both cases, the fraction of empty droplets will be the same, and thus the concentration in copies/µl will be the same in both wells (within the limits of the instrument measurement error).

At the extreme ends of the concentration range (for example, fewer than ten copies of target in a well or more than 120,000 copies of target in a well), fewer droplets in a well will lead to slightly larger error bars.

Note: For simplicity, we will use 20,000 partitions in the examples in this chapter.

Copies per Microliter

QuantaSoft software provides concentration results in copies of target per microliter (copies/µl). The dynamic range of ddPCR extends from fewer than 0.25 copies/µl to more than 5,000 copies/µl. In many cases, the fundamental quantity of interest to a user is the number of copies of target in the starting sample.

The following example shows how copies/µl is converted to copies in the starting material.

Mix 10 µl of sample with 12.5 µl of ddPCR supermix for probes and 2.5 µl of assay (primer and probe mix), for a total volume of 25 µl. Load 20 µl of this mix into a DG8™ droplet generator cartridge and run ddPCR. The software reports that the concentration is 8 copies/µl. Two equivalent methods illustrate how many total copies and how many copies/µl of the target DNA were present in the original 10 µl sample.

- Method #1: The ratio of sample to total volume is 10/25 = 2/5. Since there were 8 copies/µl in the final PCR mix, there were 8 x (5/2) = 20 copies/µl in the original sample. In the full 10 µl of the original sample, there were 10 x 20 = 200 copies of the target DNA
- Method #2: Since there were 8 copies/µl in the PCR mix and a total of 25 µl of the PCR mix was made, there were 8 x 25 = 200 copies of the target DNA in the PCR mix. This mix contained 10 µl of the original sample, so there were 200 copies of target DNA in the full 10 µl of starting sample, and 200/10 = 20 copies/µl of target in the starting sample

Copies per Droplet

A useful unit for discussing the calculations underlying ddPCR is copies of target per droplet (CPD). For illustration purposes in this chapter we will use 1 nl volume.

CPD represents a number per unit volume, and not a mass per unit volume. CPD is the average number of target copies/droplet. For a CPD of 2, for example, some droplets will have 0 copies, some will have 1, 2, 3, and so on.

There are multiple ways to calculate CPD:

- Example 1:

CPD = total number of molecules/total number of droplets

If you have 100,000 molecules total and 20,000 droplets, then

CPD = 100,000 molecules/20,000 droplets

CPD = 5 molecules/droplet

- Example 2:

CPD = (molecules/µl) x droplet volume (µl)

If you have 20 molecules in a 20 µl sample with 1 nl droplet volume, then

20 molecules/20 µl = 1.0 molecule/µl

CPD = (1.0 molecule/µl) (0.001 µl/droplet)

CPD = 0.001

Low Concentration Example

When there are far fewer molecules than partitions (for example, 500 molecules or less in 20,000 partitions), it is relatively easy to see how the ddPCR approach enables accurate quantification. Figure 3.3 shows a sample that contains six target DNA molecules. If the entire sample is converted into droplets, we will expect to find exactly six droplets that contain template, since it is statistically highly unlikely that a single droplet will start with more than one target molecule.

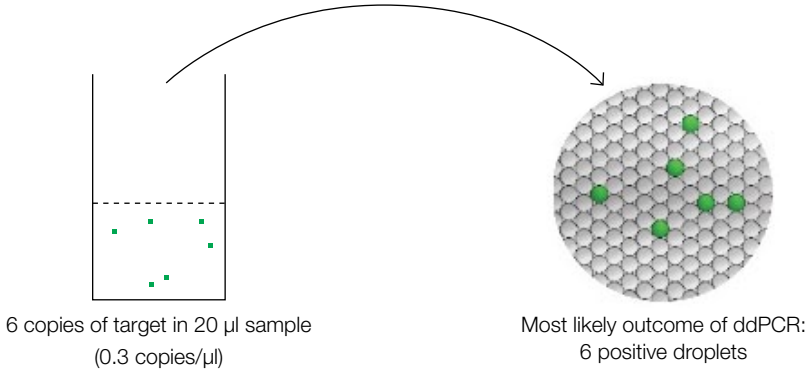


Fig. 3.3. Outcome of ddPCR in two modes of analysis.

For 20,000 droplets, the following concentration calculation is used (assumes an approximate droplet volume of 1 nl or 10^{-3} µl).

$$\text{Volume analyzed} = 20{,}000 \times 10^{-3}\ \mu\text{l/droplet} = 20\ \mu\text{l}$$

$$\text{Concentration} = \frac{\text{Copies of target}}{\text{Volume analyzed}} = \frac{6\ \text{copies}}{20\ \mu\text{l}} = 0.3\ \text{copies/µl}$$

Note: The final concentration is the same if we analyze 10,000 droplets and observe three copies of target:

$$\text{Volume analyzed} = 10{,}000 \times 10^{-3}\ \mu\text{l/droplet} = 10\ \mu\text{l}$$

$$\text{Concentration} = \frac{\text{Copies of target}}{\text{Volume analyzed}} = \frac{3\ \text{copies}}{10\ \mu\text{l}} = 0.3\ \text{copies/µl}$$

Intermediate Concentration Example

Consider the case in which there are 5,000 target molecules in 20,000 droplets (5,000 targets in 20 µl = 250 copies/µl = 0.25 CPD). Random partitioning of target molecules into droplets will lead to some droplets with 2, 3, or even 4 copies, and correspondingly more than 75% of the droplets will have zero copies. Poisson statistics tells us exactly how many droplets to expect in each category. Table 3.1 shows the expected percentage of droplets in each category. Note that in ddPCR, each droplet is distinguished as either empty/negative (zero targets) or occupied/positive (one or more targets). For the 0.25 CPD case, 78% (not 75%) of the droplets will be positive and 22% (not 25%) will be negative on average. Since Poisson statistics arise in multiple different contexts in ddPCR, we describe this phenomenon as "partitioning statistics."

Table 3.1. Expected percentage of droplets containing target DNA copies.

Targets	Droplets, %
0	78
1	19.5
2	2.4
3	0.2
4	0.01

High Concentration Example

Consider the case in which 50,000 target molecules are present in 20 µl (50,000 targets in 20 µl = 2,500 targets/µl = 2.5 CPD). With an average of 2.5 copies of target/droplet, it's not immediately obvious that there will be any empty droplets. But Poisson statistics predicts that there will be some empty droplets (1,642 empty droplets) and gives a precise relationship between the average number of copies/droplet and the expected fraction of empty droplets. Table 3.2 shows how many droplets we expect to see with 0, 1, 2, 3, 4, or more copies/droplet when there are on average 2.5 copies of target/droplet, or 50,000 copies in a 20 µl reaction volume.

Table 3.2. Expected percentage of droplets containing the target at high concentration.

Number of Target Molecules	Count of Droplets	Total Droplets, %
0	1,642	8.21
1	4,101	20.5
2	5,130	25.7
3	4,275	21.4
4	2,672	13.4
5	1,336	6.68
6	557	2.78
7	199	0.99
8	62	0.31
9	17	0.086
10	4	0.02
11	1	0.0049

Even at an average of 5 copies/droplet (the upper end of the recommended loading range), we expect to see about 134 empty droplets in a total of 20,000 droplets (data not shown).

Looking across the Whole Concentration Range

Figure 3.4 shows the number of droplets with 0, 1, 2, 3 (and so on) copies of the target DNA at different DNA concentrations. There is a simple mathematical relationship between the fraction of droplets that are unoccupied (black bar) and the concentration of target molecules. As the concentration of input target DNA increases, the expected number of unoccupied droplets decreases.

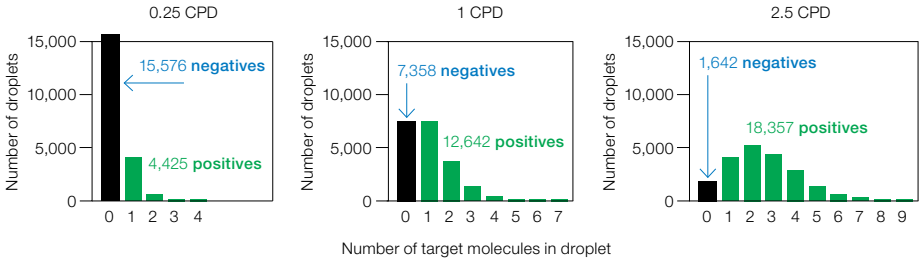


Fig. 3.4. Number of droplets with copies of target DNA at different DNA concentrations. CPD, copies per droplet.

Note: Quantification is impossible when there are no unoccupied droplets, which is approached at 8 CPD.

Concentration Calculation

Definitions

C = copies per droplet (CPD)

E = observed fraction of empty droplets

$V_{droplet}$ = volume of droplet

N_{neg} = number of negative droplets

N = total number of droplets

Formula for Calculating Concentration

The formula used by QuantaSoft software to calculate concentration is:

$$\text{Concentration} = -\ln\left(\frac{N_{neg}}{N}\right)/V_{droplet}$$

Derivation of Concentration Formula

The Poisson distribution gives probability Pr(*n*) that a droplet will contain *n* copies of target if the mean number of target copies per droplet is *C*:

$$\Pr(n) = \frac{C^n e^{-c}}{n!}$$

Inputting $n = 0$ gives the probability that a droplet will be empty for a given value of *C*:

$$\Pr(0) = e^{-c}$$

For a large number of droplets, the observed fraction of empty droplets (*E*) gives a very good estimate of Pr(0), so:

$$E = e^{-c}$$

Solving for *C* gives:

$$C = -\ln(E)$$

Recall that *C* is copies per droplet. To convert to copies per µl, divide by the droplet volume:

$$\text{Concentration} = \frac{C}{V_{droplet}}$$

Combining the two previous equations gives:

$$\text{Concentration} = \frac{-\ln(E)}{V_{droplet}}$$

Note that by definition:

$$E = \frac{N_{neg}}{N}$$

Combining the two equations above gives:

$$\text{Concentration} = -\ln\left(\frac{N_{neg}}{N}\right)/V_{droplet}$$

$$C = -\ln\left(\frac{N_{neg}}{N}\right)/V_{droplet}$$

Errors in ddPCR

Two types of errors are reported by QuantaSoft software: technical errors (Poisson errors) and total errors.

Technical errors (Poisson errors): a measurement error based on known properties of the system that can be calculated based on a single well or by pooling all the droplets from multiple wells. One of the assumptions in this error calculation is that the sample in a ddPCR well is a subsample from a larger whole. Poisson errors are an excellent estimate of technical replicate measurement errors. A technical replicate in this context is when aliquots of the same sample are loaded into multiple ddPCR wells. An interesting and useful feature of ddPCR is that it is possible to estimate the technical replicate error from a single concentration measurement.

Total errors: the recommended error to use in most applications in biology. It is the greater of the technical error and the standard error of the mean. This method is the preferred one because it prevents underestimation of the error. We can say with certainty that if you observe a standard error of the mean that is less than the theoretical technical error, you were just “lucky” and the true error is in reality at least as big as the technical error.

For experiments with replicates, both an empirical error measurement (the total error) and a theoretical technical replicate error (the Poisson error or technical error) are calculated. In most cases, it is more appropriate to report the total error measurement. If the wells are true technical replicates as defined above, the total error and the Poisson error will be nearly identical for good assays.

Note: Error bars are shown in QuantaSoft software as 95% confidence intervals. The closest parallel in quantitative PCR (qPCR) is the mean ± 2 times the standard error of the mean. Some qPCR systems by default show 68% confidence intervals, or the mean ± 1 times the standard error of the mean.

Note: The theory behind technical errors is explained in Appendix B.

4 Copy Number Variation Analysis

Overview

Analysis of copy number (CN) involves determining the number of copies of a given target locus with respect to an invariant reference locus. An alteration in copy number state with respect to the reference locus is copy number variation (CNV). CNV could be a deletion or duplication of a locus with respect to the number of copies of the reference locus (and hence genomes) present in the cell.

CNV is a prominent source of interindividual variability in the human genome, and CNV has been associated with cancers, neurological and autoimmune diseases, and adverse drug response. The major technical challenge in CN assessment is the ability to discriminate, with statistical confidence, between consecutive CN states (Figure 4.1). Fundamentally, as CN state increases, the percentage difference in target genomic material between states decreases. For example, for a given target locus, a CN of 3 is 50% more abundant in concentration per genome than a CN of 2, while a CN of 5 is only 25% more abundant per genome than a CN of 4, and so on. Current methods to analyze CNV, including single nucleotide polymorphism (SNP)–based microarrays, comparative genomic hybridization, and quantitative PCR (qPCR), lack the sensitivity and resolution needed for this fine degree of quantitative discrimination in CNV analysis.

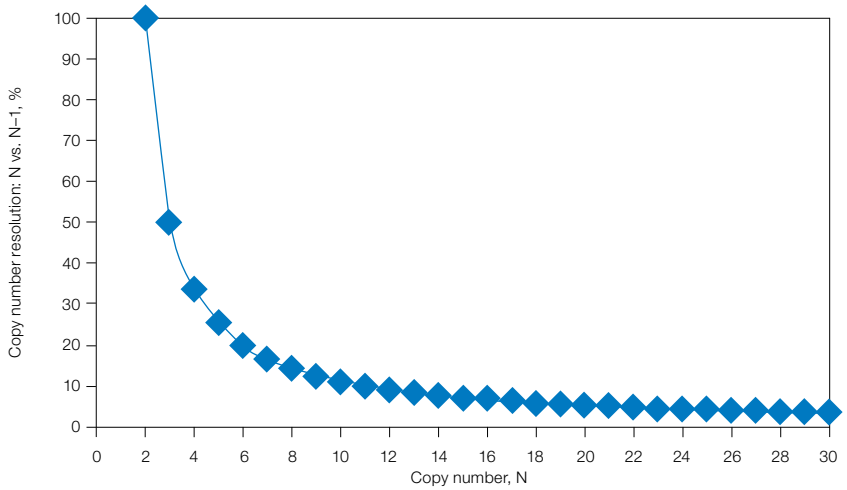


Fig. 4.1. Discrimination between consecutive CN states is more difficult at higher order copy numbers.

The massive partitioning of a CNV Droplet Digital™ PCR (ddPCR™) reaction across up to 20,000 droplets enables the fine quantitative discrimination required to resolve consecutive copy number states beyond CN 3. This is due to the high precision of the ddPCR concentration measurement and the absolute nature of the measurement. For example, a 20% difference in target concentration between a CN 5 vs. CN 6 sample can be reproducibly resolved using ddPCR (Figure 4.2). This discrimination is both a function of concentration differences between consecutive CN states, as well as the amount of DNA assayed per well.

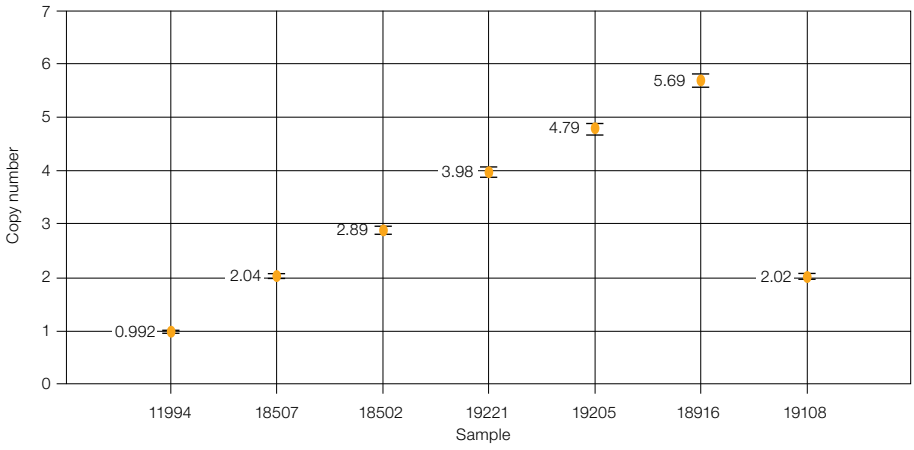


Fig. 4.2. ddPCR measurements of *MRGPRX1* copy numbers across seven Coriell DNA samples.

CNV Calculations

CNV analysis by ddPCR involves quantification of target and reference loci through the use of duplex target and reference assays. In QuantaSoft™ software, copy number is determined by calculating the ratio of the target molecule concentration to the reference molecule concentration, times the number of copies of reference species in the genome (usually 2).

$$CNV = \frac{A}{B} N_B$$

A = concentration of target species

B = concentration of reference species

N_B = number of copies of reference loci in the genome (usually 2)

The error bars on a CN estimate in QuantaSoft software are the 95% confidence interval of this measurement.

CNV Analysis in Homogeneous Samples

Analysis of CNV in homogeneous samples involves evaluation of CN state from a sample source where every cell is presumed to be identical in CN state. Examples include *CYP2D6* genotyping in patient samples, trisomy 21 detection in a clonally derived Down syndrome cell line, and counting transgene copies in a genetically modified organism (GMO) plant stock. The challenge in homogeneous samples is largely a function of the level of discrimination required at a high CN state to differentiate between adjacent CN states.

For some applications, for example, binning cancer patient samples by oncogene amplification status, discrimination between high CN states (for instance, 50 vs. 51 copies) may not be as critical as discriminating between CN states of 2 vs. 10 vs. 50.

CNV Analysis in Heterogeneous Samples

Some sample types are heterogeneous for CN state — meaning that for a given sample of cells, some have altered CN status and some do not. Examples include a breast cancer biopsy, where only a fraction of the cells are amplified for *HER2*, detection of fetal trisomy 21 from a maternal blood sample, or somatic mosaicism in normal tissue. In heterogeneous samples, the ability to accurately quantify CN alterations is a function of both the percentage of assayed cells with a CN alteration and the increase in CN state of the target gene in those cells. It is more challenging to detect CNV for a target locus in a sample where 10% of the cells have CN 3 instead of 2 than in a sample where 10% of the cells have a CN of 10 instead of 2. The problem becomes more difficult as the percentage of cells with the CN alteration decreases. Detection of CN variant cells in a heterogeneous sample is a function of both the magnitude of the CN alteration and the rarity of the altered cell (Table 4.1).

Table 4.1. Detection of CN variant cells in a heterogeneous sample.

Sensitivity Required for CN Determination in a Heterogeneous Sample			
	Diploid Copy Number		
Cells with CN Alteration, %	Wild Type	Amplified	Discrimination Needed, %
10	2	3	5
	2	10	40
	2	50	240
1	2	3	0.5
	2	10	4
	2	50	24
0.1	2	3	0.05
	2	10	0.4
	2	50	2
0.01	2	3	0.005
	2	10	0.040
	2	50	0.240

Planning CNV Experiments

Assay Design

For CNV ddPCR, target and reference assays are designed with different probe fluorophores, generally FAM-target and HEX-reference (or VIC-reference).

When possible, target and reference assays should have amplicons of similar length. This is particularly important for fragmented samples, like material extracted from formalin-fixed, paraffin-embedded (FFPE) tissue. Because CNV calculates the ratio of target concentration to reference concentration, if either target or reference amplicon site in the genome is under-represented due to fragmentation, the ratio call will be off-integer.

Check for secondary (nonspecific) products using the UCSC In-Silico PCR site (**http://genome.ucsc.edu/cgi-bin/hgPcr?command=start**). This is particularly important for CN-variable targets or members of gene families because of the presence of highly similar pseudogenes or homologs.

An ideal reference assay yields expected concentrations reproducibly and robustly across standard reaction parameters, duplex partners, and sample sources. A reference assay should be CN invariant. A good reference assay performs well across a wide range of annealing/extension temperatures.

Examples of human genome reference loci:

- *RPP30* (NCBI gene 10556)
- Ultraconserved sequences (PMID: 15131266)

Running a CNV Assay

Select **Copy Number Variation (CNV)** as the experiment type in QuantaSoft software when loading wells. Double click on the experiment name in the main software window to set the ploidy for the reference (Figure 4.3).

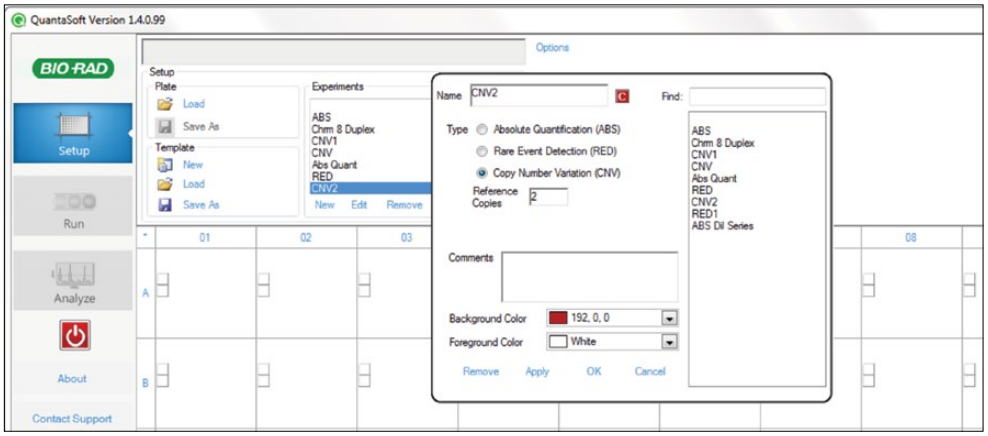


Fig. 4.3. Setting the ploidy for the CNV reference.

Note: R2 means 2 copies/genome (diploid) or 1 copy/haploid genome. If CNV experimental type is not selected for your wells, the CNV tab will not be available. This affects only the CNV charting calculations and can be applied during setup or any time after data collection.

For the reference assay, select **Reference** as type. Put this assay's concentration as the denominator, for example, "b" for the ratio chart and for the fractional abundance chart, and assign the CN selected to this assay. If both Ch1 and Ch2 are selected as "unknown," Ch1 is automatically "a" and Ch2 is automatically both "b" and the CNV reference.

Restriction Digestion

To achieve optimal accuracy in CN measurements, restriction digestion of genomic DNA is required. Restriction digestion separates tandem gene copies, ensuring proper random partitioning into droplets (Figure 4.4). Restriction digestion can also reduce sample viscosity and improve assay performance by improving template accessibility.

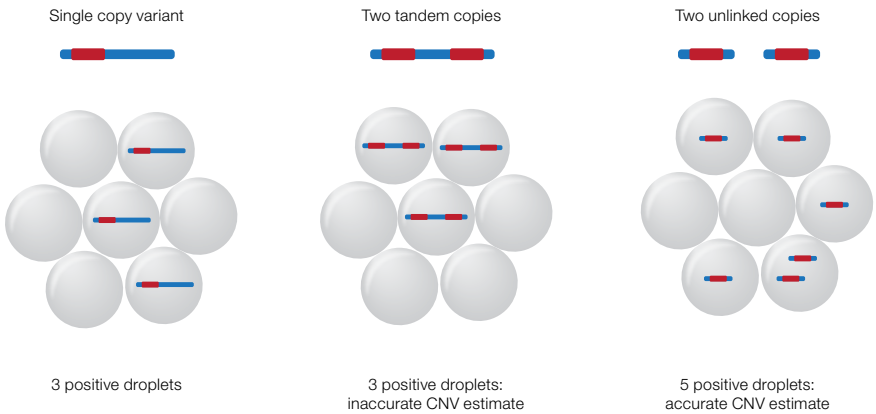


Fig. 4.4. Restriction digestion separates tandem gene copies.

Considerations in planning a restriction digestion.

- Do not cut the target or reference amplicon
- Choose a methylation-insensitive enzyme
- Read the chosen restriction enzyme FAQs on the manufacturer's website. They often describe known issues such as star activity
- For most assays, a fragment size of 5 kb or less works fine. This can typically be achieved with a 4-cutter or 6-cutter enzyme. For some assays, smaller template fragment lengths (<500 bp) are required. This may be due to nearby inhibitory secondary DNA structures/elements that get cut away with a smaller fragment size
- RestrictionMapper (**www.restrictionmapper.org**) is a website that can help you determine template fragment length and whether your designed amplicon is cleaved by a given restriction enzyme
- For human genomic DNA, use 10 U restriction enzyme/µg DNA
    - Up to 20 U enzyme/µg DNA is acceptable. Higher concentrations of enzyme could be required to resolve higher CN targets
- Some ddPCR reactions are inhibited when high volumes of digestion buffer are included because of high salt concentrations
    - If possible, avoid use of NEBuffer 3 and 3.1, which have the highest salt concentration
    - Digested templates should be diluted a minimum of 10-fold in the final ddPCR reaction setup to reduce the final salt concentration in ddPCR. For example, no more than 2 µl of a 1x digest reaction should be loaded into a 20 µl ddPCR reaction if possible
    - Many assays will perform well regardless of which NEBuffer is used or the amount of 1x digest reaction loaded into ddPCR
- Incubate the reaction for 1 hr at the recommended temperature
- Heat inactivation is not required, but can be considered if long-term storage of digested template is required. **Do not heat inactivate at greater than 65°C**
- DNA purification is not necessary after restriction digestion
- Store digested DNA at –20°C

Lists of recommended restriction enzymes for CNV ddPCR are provided in Tables 4.2 and 4.3. Conditions for a typical restriction enzyme digestion are in Table 4.4. For more information, visit the New England Biolabs, Inc. website (**www.neb.com**).

Table 4.2. Recommended restriction enzymes for CNV ddPCR (most-preferred 4-cutters).

Restriction Enzyme	Sequence	Digestion Buffer (old)	Digestion Buffer (new)	Incubation Temperature, °C
CviQI	G/TAC	NEBuffer 2, 3, BSA	3.1	25
MseI	T/TAA	NEBuffer 2, 4, BSA	CutSmart	37
AluI	AG/CT	NEBuffer 1, 2, 4	CutSmart	37
HaeIII	GG/CC	NEBuffer 2, 4	CutSmart	37
BsmI	GAATGC(1/–1)	NEBuffer 4	CutSmart	65
BstYI	R/GATCY	NEBuffer 2,4	2.1	60

BSA, bovine serum albumin.

Table 4.3. Recommended restriction enzymes for CNV ddPCR (most-preferred 6-cutters).

Restriction Enzyme	Sequence	Methylation Sensitivity	Digestion (old)	Digestion Buffer (new)	Incubation Temperature, °C
HindIII	A/AGCTT	None	NEBuffer 2	NEBuffer 2.1	37
EcoRI	G/AATTC	CpG	NEBuffer 1, 2, 3, 4	EcoRI buffer	37

NEB, nuclear extraction buffer.

Table 4.4. Conditions for a typical restriction enzyme digestion.

Reaction Component	Final Concentration	Volume (50 µl/reaction), µl
10x digestion buffer	1x	5
gDNA	100 ng/µl	Variable
Restriction endonuclease	10 U/µg DNA	Variable
100x BSA (as needed)	1x	0.5
Water	—	Variable

BSA, bovine serum albumin; gDNA, genomic DNA.

Newly designed assays should be run across a thermal gradient (for example, 65–55°C) to identify an annealing/extension temperature that optimizes separation between positive and negative droplets while minimizing rain (droplets that fall between the major positive and negative populations). If possible, select an annealing/extension temperature that optimizes performance of both target and reference assays.

DNA Loading for Lower-Order CN Analysis (diploid CN <10)

For most routine CNV ddPCR applications, where a diploid target CN is expected to be 10 or less, approximately 0.2–1.0 reference gene copies per droplet (CPD) of DNA sample should be loaded per well. This corresponds to 10–66 ng of human genomic DNA per well.

DNA Loading for Higher-Order CN Analysis (diploid CN >10)

When target CN is expected to exceed 10 copies/diploid genome, <15 ng of human genomic DNA should be loaded per well. This ensures both target and reference copies fall within the dynamic range of the instrument (Table 4.5). For example, if a reference locus is loaded at 1 CPD (1,000 copies/µl), a target present 20 times more frequently per genome is effectively loaded at 20 CPD (20,000 copies/µl), which is beyond the range of accurate quantification of the instrument. If a reference locus is loaded at 0.2 CPD (200 copies/µl), a target present 20 times more frequently is loaded at 4 CPD (4,000 copies/µl), which is within the dynamic range of the instrument. For diploid CN >50, strategies where multiple wells are used to estimate target and reference concentrations can be used.

Table 4.5. Sample loading considerations for higher-order CN targets (10–50 copies/diploid genome).

Reference Loading for CN 2, CPD	Reference, copies/µl	Target Loading for CN ≥10		
		Target, copies/µl		
	CN 2	CN 10	CN 20	CN 40
1	1,000	5,000	10,000	20,000
0.2	200	1,000	2,000	4,000

CPD, copies per droplet; CN, copy number.

5 Rare Mutation and Sequence Detection

Overview

Droplet Digital™ PCR (ddPCR™) enables detection and analysis of nucleic acids at a level of sensitivity and precision beyond the capabilities of previous methods. Applications that focus on the lower limits of nucleic acid detection can be separated into two classes:

- Rare mutation detection
- Rare sequence detection

Rare mutation detection (RMD) and rare sequence detection (RSD) can be classified according to their assay components. An RMD assay is made up of a single primer set plus two competitive probes (Figure 5.1). RSD uses either a single assay to detect a rare sequence per unit volume (Case 1) or two independent, noncompetitive assays to detect a rare sequence as a proportion of a more common sequence (Case 2). Both RMD and RSD take advantage of the high sensitivity of ddPCR.

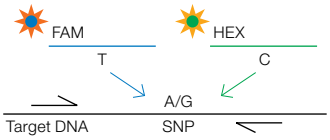
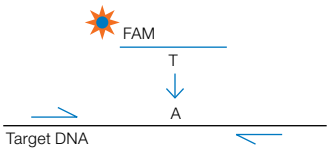
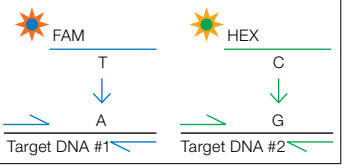
	Measurement Units	Examples	Assay Components	Assay Schematic
Rare Mutation Detection				
	Ratio (% or a/[a+b])	■ Tumor biopsy: 0.01% mutant detected, or 1 in 10,000 ■ Single nucleotide polymorphisms (SNPs) ■ Small indels	1 primer pair, 2 competitive probes	
Rare Sequence Detection				
Case 1	Copies/volume	■ Invasive species monitoring (copies/ml lake water) ■ Human immuno-deficiency virus (HIV) detection (copies/ml plasma)	1 assay (1 primer/probe set)	
Case 2	Ratio (% or a/[a+b]) or copies/unit reference	■ Gene expression of rare transcripts ■ Viral staging ■ Indels	2 independent assays (2 primer/probe sets)	

Fig. 5.1. Rare mutation detection and rare sequence detection assays.

For any rare detection assay, standard design rules should be used in the design of TaqMan probes and primer sets (see Chapter 2). In general, primers should be designed to have melting temperatures (T_m) of ~60°C (1 M NaCl, 1 µM concentration) and should be within 2°C of each other. Furthermore, the probe must have a melting temperature 3–10°C higher than the primer T_m. A temperature binding enhancer, such as a locked nucleic acid (LNA) can be used to shorten the number of nucleotides in the probe while maintaining a higher T_m. Finally, the mutant site should be positioned near the middle of the probe sequence.

Rare detection assay designs should be validated with a temperature gradient to ensure the highest specificity between the mutant and wild-type clusters. The optimal annealing temperature is defined by the condition in which the mutant probe exhibits no false positives in the wild-type–only sample and the relative distance between the FAM-only (mutant) and HEX (or VIC)-only (wild-type) clusters is maximal.

To enable the ultrasensitive detection of mutant targets, very high loads of DNA are required. However, for ddPCR, amounts of intact human DNA exceeding 66 ng (20,000 genome equivalents)/20 µl reaction negatively affect the accuracy of DNA quantification. To mitigate this effect, gDNA must be fragmented by restriction digestion using an enzyme that cuts around the amplicon(s) of interest. Once fragmented, the human gDNA concentration can exceed 1 µg/20 µl reaction without affecting DNA quantification.

Rare Mutation Detection

Rare mutation detection occurs when a biomarker exists within a background of a highly abundant counterpart that differs by only a single nucleotide. Many methods for mutation analysis have poor selectivity and fail to detect mutant sequences with abundances of less than one in 100 wild-type sequences (Scott 2011, Benlloch et al. 2006, Whitehall et al. 2009). If enough DNA sample is available for testing, a limit of detection of 1 mutant in 100,000 wild type can be detected using a well-designed assay and proper experimental setup.

ddPCR for Rare Allele Detection and Experimental Considerations

The partitioning effect, which is a hallmark of ddPCR technology, has an important impact on the sensitivity and specificity of a PCR reaction. For applications reliant on measuring a low-abundance rare mutant allele in a large excess of wild-type DNA, partitioning the sample into droplets increases the sensitivity by orders of magnitude by effectively diluting away the background. This means that the mutant target is present in droplets at a much greater relative abundance than it would be in bulk solution. Considering a rare allele detection scenario where the desired mutant has an abundance of 0.1% relative to wild-type DNA, Figure 5.2 shows that randomly partitioning the sample into 20,000 1-nl droplets increases the relative abundance by 330-fold, resulting in drastic improvement of the achievable sensitivity compared to real-time PCR.

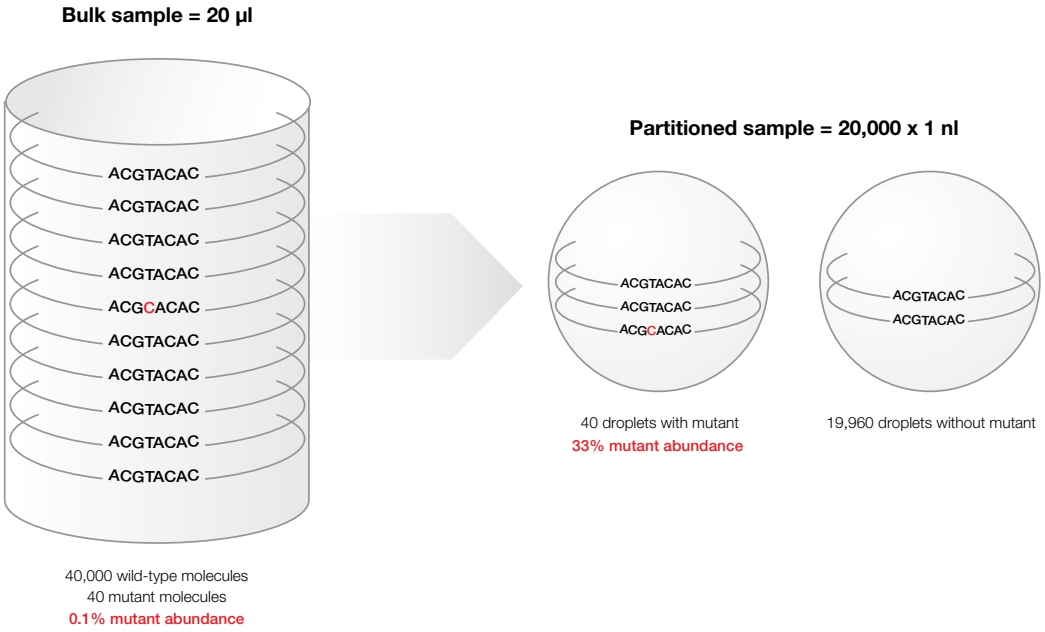


Fig. 5.2. Effect of partitioning on the relative abundance of a mutant target in an excess of wild-type DNA.

Figure 5.2 depicts a bulk 20 µl solution containing 40 mutants and 40,000 wild-type DNA molecules with a mutant abundance of 0.1%, which is beyond the limit of quantification by real-time PCR. After random partitioning into 20,000 1-nl droplets, there are 40 droplets containing target molecules at a relative mutant abundance of 33%, which are easily distinguishable from the 19,960 droplets containing wild-type molecules only.

RMD Experiment Considerations

The first consideration for low-level detection is the amount of DNA available. If 1 mutant in 100,000 wild-type sequences, or 0.001%, is to be detected, then statistically at least 300,000 haploid genomes must be screened. For human DNA this is 1 µg of DNA.

The challenge for RMD assay development is that it must discriminate between two highly similar sequences, one of which is significantly more abundant than the other. An example of an RMD assay is detection of a single nucleotide polymorphism (SNP) in a cancer biopsy. An RMD assay comprises a single set of primers plus two competitive probes (each probe with a different fluorophore), one detecting the wild-type allele, and one detecting the variant allele.

Testing an RMD Assay

RMD assays should be tested for specificity by first running a temperature gradient at a relatively high wild-type DNA concentration and then by running a concentration gradient of wild-type DNA with a spike-in of mutant DNA to assess specificity.

First, run a temperature gradient using both a row/column of restriction digested, 100% wild-type DNA at a concentration between 2,000–5,000 copies/µl previously restriction digested and a row/column of 2,000–5,000 copies/µl wild-type DNA including 50–200 copies/µl mutant DNA. Using the 1-D amplitude plot, select the T_m with no false positives, good separation between positive and negative populations, and minimal probe cross-reactivity (Figure 5.3). Also, be sure to simultaneously check the concentration plots to make sure the concentration is the expected result at the temperature selected.

In Figure 5.3, wells A–H are an annealing/extension gradient from 65–55°C, respectively. Panel A is a 1-D amplitude plot showing the FAM-labeled mutant assay results from a temperature gradient on the SNP *PI3Kca_E545K*. Wells A03–H03 contain 100% wild-type DNA, and A04–H04 contain 50% wild-type/mutant DNA. Well D was selected at an annealing/extension temperature of 61.4°C. At lower annealing/extension temperatures, false positives appear. Panel B is a 1-D amplitude plot of FAM-labeled mutant assay results of *PI3Kca_H1047R*. Wells A06–H06 contain 100% wild-type DNA, and A07–H07 contain 50% wild-type/mutant DNA. Well B was selected at an annealing/extension temperature of 64.5°C. At lower annealing/extension temperatures, the positive clusters for the VIC channel have increasing probe cross-reactivity with the nonspecific target, causing the wild-type droplets to have some level of FAM fluorescence in the FAM channel.

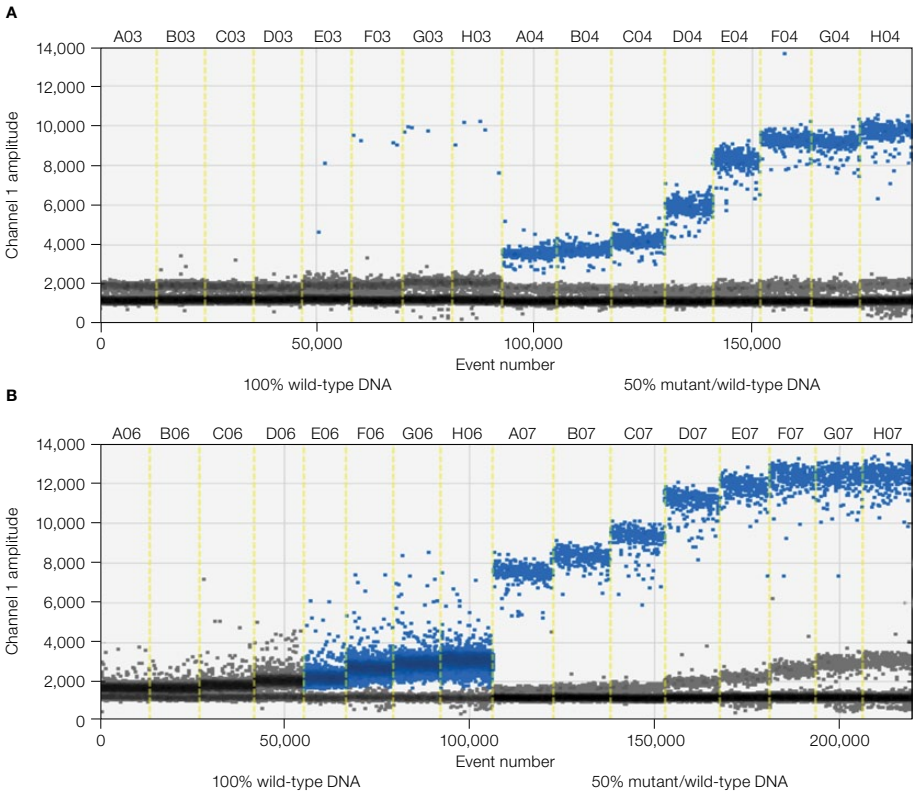


Fig. 5.3. Two examples of SNP assay temperature gradients. A, 1-D amplitude plot of the FAM-labeled mutant assay results of *PI3Kca_E545K*; **B**, 1-D amplitude plot of the FAM-labeled mutant assay results of *PI3Kca_H1047R*.

Once the best melting temperatures have been identified using 1-D plots, use 2-D amplitude plots to assess assay sensitivity. Look for no false positives or "spray" of droplets into the double-positive region in the 2-D amplitude plot of the wild-type–only samples.

The 2-D amplitude plot for the SNP assay should have no positive droplets in the double-positive region in the wild-type–only well. Wild-type–only control wells should have the same concentration of DNA as the highest concentration experimental well. Figure 5.4 shows an example of false positives or spray into the double-positive region. For more sensitive mutant detection, assay redesign may be recommended to resolve this.

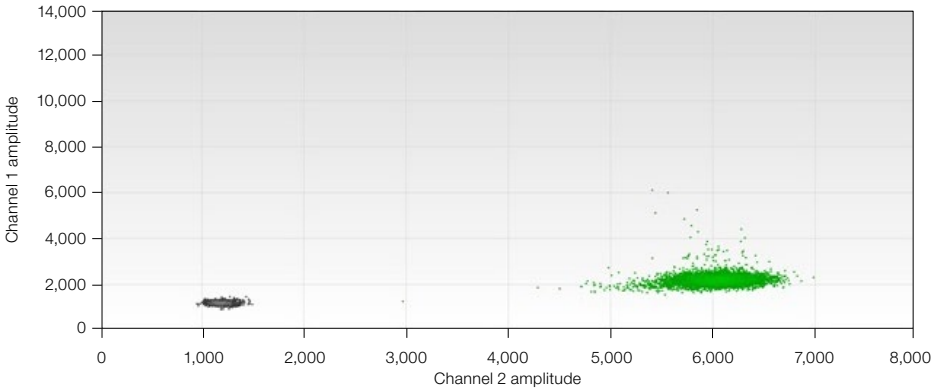


Fig. 5.4. 2-D amplitude plot of a wild-type control well containing 2,000 copies/µl of an SNP assay with spray of false positives into the double-positive region of the plot.

Figure 5.5 shows a 2-D plot of a single well with an assay for SNP *KRAS G12V* that indicates no false positives at 5,000 copies/µl. This assay can detect 0.001% mutant, or 1 mutant in 100,000 wild-type targets.

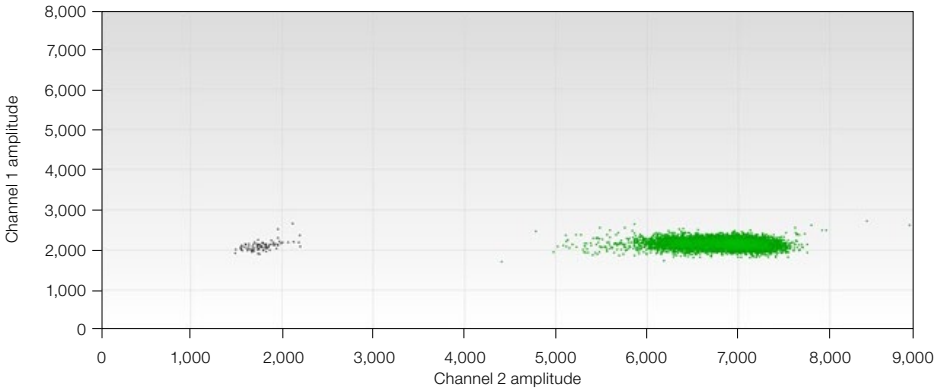


Fig. 5.5. 2-D amplitude plot of a wild-type control well containing 5,000 copies/µl of an SNP assay with no spray of false positives into the double-positive region of the plot.

Figure 5.6 shows a 2-D amplitude plot of four combined or merged wells of SNP *KRAS G12V* detecting 0.002% mutant.

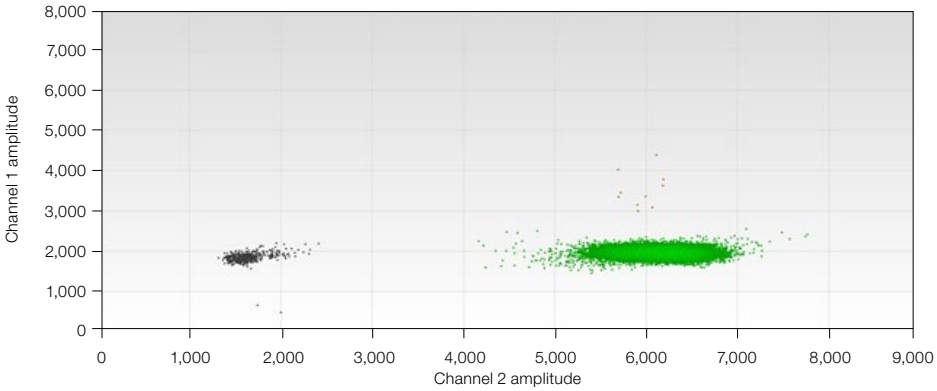


Fig. 5.6. 2-D amplitude plot showing 0.002% mutant detection in four merged wells.

Interpreting 2-D Plot Results for SNP Assays

How does orthogonality affect assay performance? The shift of single-positive clusters toward the double-positive region makes it more difficult to separate single-positive droplets from double-positive droplets.

In Figure 5.7, the four clusters represent the droplets as follows:

- Blue cluster (top left), FAM-positive droplets with only mutant template
- Brown cluster (top right), double-positive droplets with both templates inside
- Gray cluster (bottom left), negative droplets with no template
- Green cluster (bottom right), VIC-positive droplets with only wild-type template

The orthogonality of the 2-D pattern means the four clusters are located spatially at right angles to each other. In an RMD assay, clusters are commonly deviated from the corner (right angles). For example, the blue cluster is shifted to right relative to the negative cluster in Figure 5.7, a result of wild-type probe (labeled with VIC) cross-reacting with mutant amplicon. Also, the double-positive cluster in an RMD assay is usually located between the two single-positive clusters rather than at the upper-right corner, due to cross-reactivity.

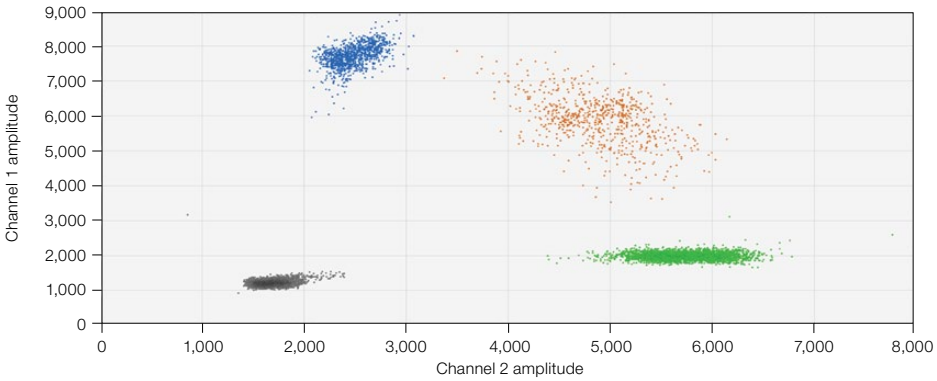


Fig. 5.7. 2-D plot of *BRAF V600E* assay.

Statistical Considerations for Rare Detection Experimental Design

The limit of detection (LoD) is the minimum concentration of the mutant (rare) sequence that can be reliably differentiated from a negative control (100% wild type). In RMD, LoD is typically quoted as a ratio or a percentage: for example, 1 in 10,000, or 0.01%. In quantitative PCR, the LoD is largely a function of the cross-reactivity of the probes. In contrast, in ddPCR, the LoD is determined primarily by the number of wild-type molecules that are screened. The LoD can be adjusted to the precise requirement of any application by adjusting the number of wells, and therefore molecules, screened.

Consider a sample at a mutant or rare sequence concentration of 1 mutant or sequence in 10,000 wild type. To guarantee with 95% confidence that at least 1 mutant molecule will be screened in this sample, statistics dictates that at least 30,000 wild-type molecules must be screened, or three times the number of expected wild-type target molecules. This is about 100 ng/well of human DNA where the target has a copy of 1/haploid genome (Table 5.1).

# of molecules to screen = 3 × background molecules

Additional wells may be screened to ensure detection of more than one positive droplet. For ddPCR, the ability to merge multiple wells into a meta-well provides more experimental flexibility to tune the experiment to the required LoD. The number of cells or amount of DNA needed to screen a given number of background molecules is described in Table 5.1. The number of wells needed is a conservative estimate. The row in bold reflects the best performance in one well. Researchers may choose to screen additional wells to ensure detection of more than one positive droplet.

Table 5.1. Requirements for different LoDs for RMD, assuming an ideal assay with a droplet false-positive rate of zero.

	Required Starting Material (human)			
LoD	**Total Copies to Screen**	**Diploid Cells**	**Amount of DNA**	**Number of Wells**
1 in 1,000	3,000	1,500	0.010 µg	1
1 in 10,000	30,000	15,000	0.10 µg	1
1 in 25,000	**75,000**	**37,500**	**0.25 µg**	**1**
1 in 100,000	300,000	150,000	1.0 µg	4
1 in 1,00,000	3,000,000	1,500,000	10.0 µg	40

Recommended Controls

If a single wild-type or negative sample control well is run and the observed positive droplets are zero, then it is good practice to require at least three positive droplets in order to call a sample positive. The three positive droplets can be in either a single well or across merged wells. On the other hand, if you run a full plate of negative sample control wells and observe zero positive droplets, consider making a positive call based on a single positive droplet. However, we don't usually recommend this because it may be difficult to control for handling or contamination issues. If the same sample is spread across multiple wells, then the number of positive droplets should be pooled across all the wells and a similar number of negative control wells should be run.

Our guidelines assume that the number of positives in no template control (NTC) wells is also zero, which indicates good handling practices. If these numbers are not zero, the rate of potential false positives must be accounted for in calling a positive sample. Control wells may not be zero for the rare sequence. This could mean that contaminating template DNA is in the reaction wells. If positive droplets in the NTC wells occur, make sure that good laboratory practices for PCR are being followed in the laboratory (see Kwok and Higuchi 1989).

To limit workspace contamination, wipe down your pipets, tip boxes, and benchtops with 5–10% bleach, prepare master mixes in a template-free environment, add samples and generate droplets in an amplicon-free environment, read droplets in a room separate from the sample preparations, do not reuse DG8™ droplet generator cartridges, oils, gaskets, plates, or pipet tips, and wear appropriate personal protective equipment that is discarded or confined to appropriate locations.

In addition to workspace contamination, consider careful experimental setup procedures before droplet generation. Be careful about anything that might produce aerosolized DNA. We recommend mixing your sample by pipetting up and down the full volume 10–12 times rather than vortexing or centrifuging your plate because removing the seal after these processes can spray contaminating DNA into other wells.

We recommend always running negative controls to monitor for all possible sources of false-positive droplets, given the risk of laboratory contamination when working with templates and amplicons. With an extremely sensitive technology such as ddPCR, we recommend running enough negative sample controls as part of every experiment so that you can demonstrate that the probability of calling a true negative sample incorrectly is below a certain threshold.

Experimental Strategies for RMD

Once the number of target molecules to be screened has been determined for a given LoD, the number of wells required to screen that number of targets must be determined. In the QX100™ or QX200™ Droplet Digital PCR system, up to 1 µg of DNA can be loaded into a single well with a recommended limit of 5,000–6,000 copies/µl per well. To achieve a lower LoD than 1 in 25,000 using human DNA (with a copy number of one per haploid), more wild-type molecules can be screened by running the same sample in multiple wells.

Factors that Impact RMD Calculations

Table 5.1 outlines RMD experimental design based on statistical requirements. Additional real-world factors will impact these numbers in an assay-specific and sample-specific way and should be considered when planning an RMD experiment.

- Sample availability — depending on the source (formalin-fixed, paraffin-embedded [FFPE] or fresh frozen), a typical tissue biopsy will provide <250 ng of sample, meaning <75,000 genome copies to screen and at best a 1 in 25,000 LoD. With a more typical 100 ng of DNA from a biopsy, an LoD of 1 in 10,000 is more practical
- Sample integrity and preparation — the calculations above assume that every target present can be successfully assayed and does not account for nonamplifiable DNA. Samples can have varying degrees of degradation and chemical damage. FFPE samples, for instance, are routinely used in cancer research, but can provide DNA of poor quality. Consider this impact when calculating the amount of starting material needed to effectively screen the desired number of target molecules

Sample Preparation

To achieve optimal accuracy in copy number measurements, restriction digestion of genomic DNA is required. Restriction digestion separates tandem gene copies, ensuring proper random partitioning into droplets. Restriction digestion can also reduce sample viscosity and improve assay performance by improving template accessibility.

Rare Sequence Detection

An RSD application might require one or two assays. If the goal is to quantify the amount of target sequence in a given volume of starting material, a single assay is used (Figure 5.8). If the goal is to quantify the amount of target sequence with respect to a given reference sequence, two independent assays are required (Figure 5.9). In either case, assay design and optimization for RSD has less risk of cross-reactivity of the target assay with background DNA.

RSD Experimental Strategies

Some RSD applications require reliable quantification of rare sequences while others require detection of a rare sequence. This difference dictates the lower bounds of sensitivity for a given assay and application. For RSD, the LoD can be defined either in terms of the total volume of material analyzed or in terms of the number of copies of some type of background DNA.

In RSD, absolute quantification of the target sequence is often required. In general, ddPCR can provide an accurate quantification of a rare target sequence at a low concentration. This eliminates the need for absolute standards and standard curves and improves reproducibility across experiments and laboratories. In addition, in ddPCR, small variations in PCR efficiency between wells have no effect on the measured concentration.

When absolute quantification is applied to an RSD application, an important consideration is the limit of quantification, or LoQ, which is the lowest concentration you can reliably measure within a predetermined variance, or coefficient of variation (CV). For example, if you want to quantify within 20%, the LoQ is the lowest concentration with which you can reliably measure to within ±20% of the real value. For RSD, to reliably detect 1 in 100,000 cells, at least 300,000 background cells must be screened. Similarly, to achieve an LoD of 1 in 1 ml of sample, 3 ml of sample must be screened.

Two different experimental setups are recommended for RSD applications, depending on whether detection is with respect to a starting volume of sample or with respect to a second background target that requires quantification.

Case 1: Quantification with Respect to Total Starting Volume

To detect foreign DNA in 5 ml of lake water, 15 ml of lake water must be screened. The experiment is shown in Figure 5.8 for the case of a single ddPCR well. Depending on how much total DNA is extracted from the 15 ml sample, more than one well may be needed to analyze the sample because up to 2.5 µg of total DNA can be loaded into one reaction well.

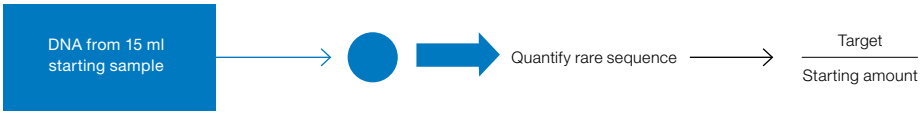


Fig. 5.8. Strategy for detecting rare foreign DNA in a sample of defined volume.

Case 2: Quantification with Respect to Second DNA Sequence

To detect a very low target concentration in a high background sample, for example 1 copy of virus/100,000 peripheral blood mononuclear cells (PBMCs), the sample can be analyzed at two different concentrations (Figure 5.9). The high-concentration wells provide sensitive detection of the rare sequence while the low-concentration wells enable quantification of the background sequence. For example, to reliably detect an average of 1 virus in 100,000 PBMCs, we must screen 300,000 PBMCs, or 2 µg of DNA (Table 5.1).

This requires three wells on the QX100 system: two wells at high concentration to screen enough PBMCs for the presence of rare virus, and a third well at a lower concentration to quantify the PBMCs. This experimental setup is shown in Figure 5.9.

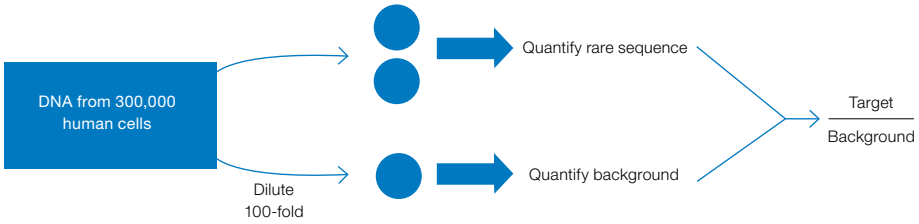


Fig. 5.9. Detection of a very low target concentration in a high background (such as a low viral load in a given number of PBMCs) by analyzing samples at two different concentrations.

The target sequence (the "rare" sequence) might be quantified at either concentration, depending on the amount present in the starting sample. The background sequence is quantified in the low concentration well.

In the examples shown in Table 5.2, the LoD is defined with respect to the number of human cells. The number of wells needed is a conservative estimate that considers that droplets can be lost due to workflow or quality control specifications.

Table 5.2. Case 2: Experimental setup for different LoD and LoQ in rare sequence detection.

				Number of Wells	
LoD	LoQ (<10% CV)	Human Diploid Cells	Amount of DNA, µg	High Concentration	Low Concentration
1 in 5,000 cells	100 in 15,000 cells	15,000	0.10	1	0
1 in 12,500 cells	100 in 37,500 cells	37,500	0.25	1	0
1 in 100,000 cells	100 in 300,000 cells	300,000	2.0	2	1
1 in 500,000 cells	100 in 1,500,000 cells	1,500,000	10.0	10	1

In the example given, the measurement error (CV) is introduced at the time of subsampling (at the time of the blood draw), not by the measurement system. Poisson statistics dictate that if *N* target molecules are measured in a sample, the standard deviation of the measurement is roughly the square root of *N*. In this example, if 100 copies of virus are present in the sample, the standard deviation of the measurement is 10 and the CV is 10/100 = 10%. This means that we can accurately quantify, to ±10%, 100 or more copies of virus in 100,000 PBMCs. No detection system can do better than this theoretical limit.

Factors that Impact RSD Calculations

- False-positive rate — the false-positive rate must be measured and considered when designing experiments to achieve very low LoD or accurate quantification
- Sample integrity and preparation — it may be useful to spike a PCR control into the original sample and use a second assay in the same well to measure its level. This enables you to control for PCR inhibitor and sample preparation failures
- Pipetting accuracy in dilutions — errors introduced by pipetting may contribute to the overall accuracy of the measurement

References

Benlloch S et al. (2006). Detection of BRAF V600E mutation in colorectal cancer: Comparison of automatic sequencing and real-time chemistry methodology. J Mol Diagn 8, 540–543.

Kwok S and Higuchi R (1989). Avoiding false positives with PCR. Nature 339, 237–238.

Scott LM (2011). The JAK2 exon 12 mutations: A comprehensive review. Am J Hematol 86, 668–676.

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6 Gene Expression

Overview

Reverse transcription quantitative PCR (RT-qPCR) is a commonly used method in gene expression studies. It is straightforward, sensitive, and has a wide dynamic range. There are two types of approaches for the RT-qPCR reaction: one-step and two-step RT-qPCR.

One-Step RT-qPCR

One-step RT-qPCR simplifies the reaction setup by combining the first-strand cDNA synthesis (reverse transcription) and qPCR in one mixture. It also greatly reduces the possibility of contamination by eliminating the cDNA-to-PCR operation step. One-step RT-qPCR can use only a limited number of probes per sample, but because it amplifies the whole sample, the sensitivity is greatly enhanced. The disadvantage of one-step RT-qPCR is that it is less amenable to multiplex assays and allows for less flexibility in priming strategies.

Two-Step RT-qPCR

Two-step RT-qPCR performs the first-strand cDNA synthesis reverse transcription and qPCR in separate mixtures. This method allows for the measurement of multiple messages from a single RNA sample. It also enables you to use different PCR reaction conditions and priming methods.

To measure gene expression with PCR, you must first convert the RNA into DNA by reverse transcription. There are three types of primers that may be used in reverse transcription:

- Oligo(dT) — priming with oligo(dT) results in cDNA synthesis that is biased to the 3' end of polyadenylated transcripts and will create only cDNA from mRNA templates
- Random primers — random priming is not subject to end bias and is not limited to mRNA, but it is sensitive to the sequence and secondary structures of the template
- Sequence-specific primers — sequence-specific primers can be designed adjacent to the PCR target, but the ability to perform multiplex qPCR from an individual sample is limited

Similar to RT-qPCR, reverse transcription Droplet Digital™ PCR (RT-ddPCR) approaches can also use a one-step or two-step protocol. Choose your approach based on your experimental purpose.

Two-Step Reverse Transcription ddPCR

Obtain RNA

Use a commercial kit to extract RNA. Store at 100 ng/µl in 1/10 TE buffer (0.1x TE) or other appropriate buffer at –80°C.

Generate cDNA

Generate cDNA according to standard protocols. We recommend Bio-Rad's iScript™ Select cDNA synthesis kit for oligo(dT) or gene-specific priming. For random priming, we recommend the iScript advanced cDNA synthesis kit for RT-qPCR. Follow the instructions in the manual of the respective cDNA kit.

Once the reverse transcription is complete, reduce the concentration of cDNA to about 0.2 ng/µl RNA equivalent and use 5 µl per Droplet Digital PCR (ddPCR™) reaction (total volume 20 µl). Typically 1 ng (5 µl of 0.2 ng/µl) of RNA-equivalent cDNA per ddPCR reaction is adequate to measure most of the transcripts reliably. If the RNA is highly degraded or very low quality, as is the case with RNA from formalin-fixed, paraffin-embedded (FFPE) samples, or the transcript of interest is expected to be found at less than 1 copy/cell, such as a transcript from a cancer cell in a large background of normal cells, the previous guideline of 1 ng/reaction no longer holds. Under these situations, up to 10 µl of the RT reaction can be added to one ddPCR reaction without altering its performance.

One-Step RT-ddPCR Kit for Probes

The one-step RT-ddPCR kit for probes follows the same workflow as the ddPCR supermix for probes, with the benefit that you can now directly partition sample RNA instead of DNA. The sample is partitioned into 20,000 droplets, with target and background RNA randomly distributed among the droplets. An RNase inhibitor included in the formulation minimizes template degradation during reaction setup and droplet generation. After reverse transcription, the resulting cDNA is amplified for target detection using TaqMan hydrolysis probes. After PCR amplification, each droplet provides a fluorescent positive or negative signal indicating the target RNA was present or not present after partitioning. Each droplet provides an independent digital measurement. Positive and negative droplets are counted and software calculates the concentration of target RNA as copies/µl.

The one-step RT-ddPCR kit for probes is formulated for efficient and sensitive reverse transcription over a wide linear dynamic range of input RNA for ddPCR. Use a commercial kit to extract RNA, then store RNA at 100 ng/µl in 1/10 TE buffer (0.1x TE) or other appropriate buffer at –80˚C. A unique hot-start reverse transcriptase enables convenient reaction setup. The reverse transcription reaction is performed at 55–60°C, enhancing the specificity and efficiency of primer-mediated cDNA conversion. The thermostable enzymes allow RNA template to be reverse transcribed and subsequently amplified in the same reaction tube. Template-specific primer annealing at elevated temperatures significantly improves stringency and melting of secondary structures.

Data Analysis

In QuantaSoft™ software, create a plate map by designing a new plate with a layout according to your experimental design. Designate the experiment type as Absolute Quantification (ABS) in the Well Editor window during plate setup. Designate which assay is the target and which is the reference assay (Channel 1 or 2) in the Well Editor window.

If the plate was set up for ABS analysis, automatic thresholding will determine concentrations and populate the data tables in the analysis mode of the software. To get the ratio of target over reference for gene expression, a duplex assay must be run with two different probes (FAM and HEX [or VIC]).

In QuantaSoft data analysis, a button labeled Ratio will become active if the experiment contains a duplex assay set up with a FAM assay and a HEX or VIC assay (Figure 6.1). Clicking on the **Ratio** button plots the relative ratio of a/b (the concentration of the FAM assay over the concentration of HEX or VIC assay). You can also click the **Inverse** button (Figure 6.1) if you switched the assay dyes where FAM is the reference and HEX or VIC is the target (the inverse makes the ratio b/a instead of a/b).

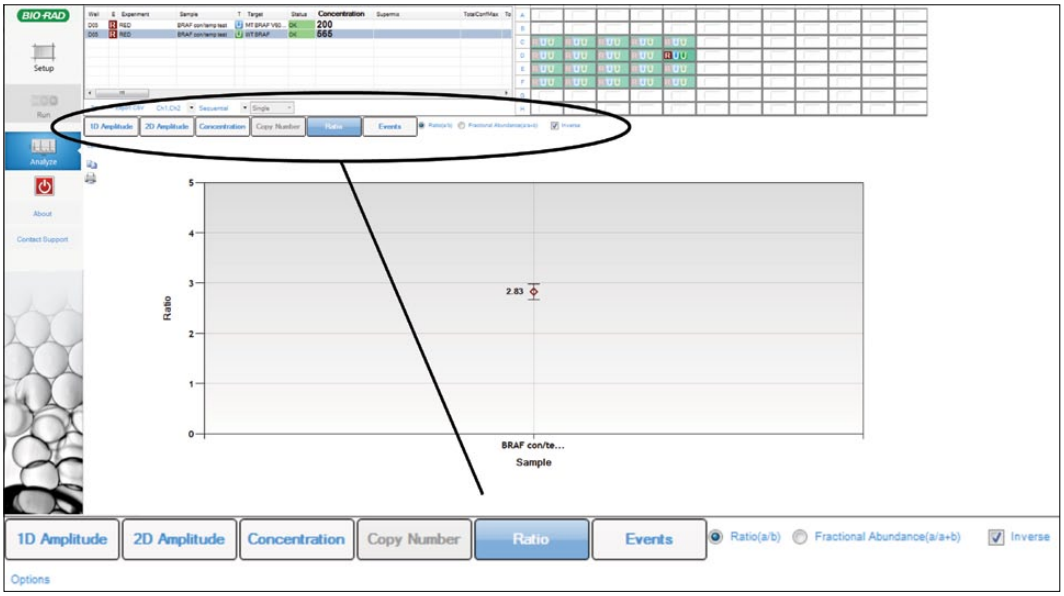

Fig. 6.1. QuantaSoft software ratio feature and inverse checkbox for gene expression ratios.

ddPCR Gene Expression Data

As shown in Figure 6.2, cDNA copies were made on three in vitro transcribed RNAs with the one-step RT-ddPCR kit for probes or two-step RT-qPCR with iScript advanced cDNA synthesis kit for RT-qPCR. The data are generally comparable on most of the assay locations. A few sites are much lower in one-step RT-ddPCR because those sites may be involved in secondary structures and the priming sites might not be accessible to the reverse transcription enzyme. You must decide whether one-step or two-step RT-ddPCR is best for your experiment.

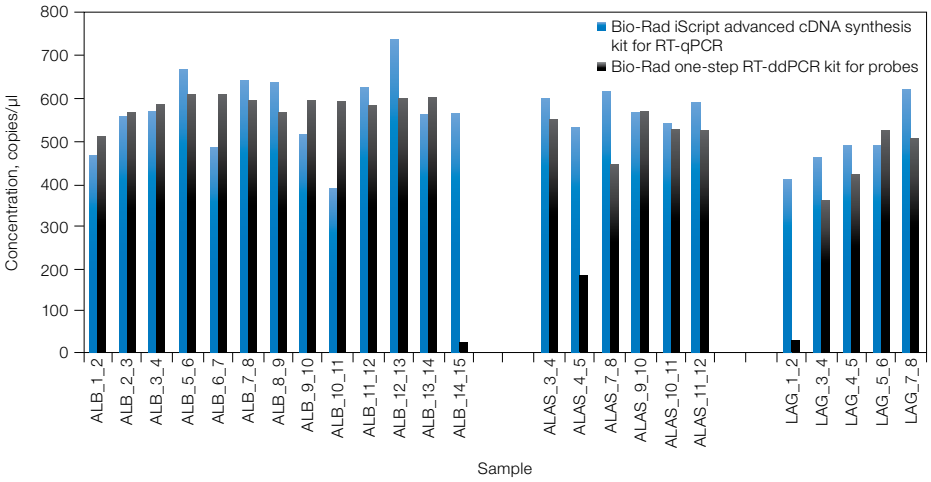

Fig. 6.2. Comparison of one-step RT-ddPCR and two-step RT-ddPCR with in vitro transcribed RNAs spiked in Ambion human brain reference RNA.

HER2 Study

The human epidermal growth factor receptor 2 gene (*HER2*) is encoded by the *ERBB2* proto-oncogene and is involved in signal transduction for cell growth and differentiation. It is a cell surface receptor for tyrosine kinase and a proto-oncogene. Overexpression of *ERBB2* is of clinical relevance in breast cancer because of its prognostic value in correlating elevated expression with worsening clinical outcome. *ERBB2* assessment is also important because successful antitumor treatment with Herceptin is strongly correlated with *ERBB2* overexpression in the tumor (about 30% of all breast tumors overexpress *ERBB2*).

Data Analysis Results

Results demonstrate that ddPCR can be used to assess expression levels of *HER2* transcripts in FFPE human breast tissues samples that were concurrently assessed via pathologists using fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC) methods.

Figure 6.3 illustrates the determination of *ERBB2* transcript levels relative to the *EEF2* reference gene. The "best coverage" *ERBB2* assay was tested with four fresh-frozen breast tumor RNA samples (OriGene Technologies, Inc). FAM (*ERBB2*) concentration, VIC (*EEF2*) concentration, and normalized *ERBB2* to *EEF2* concentrations are shown in Figure 6.3 (blue, green, and maroon, respectively).

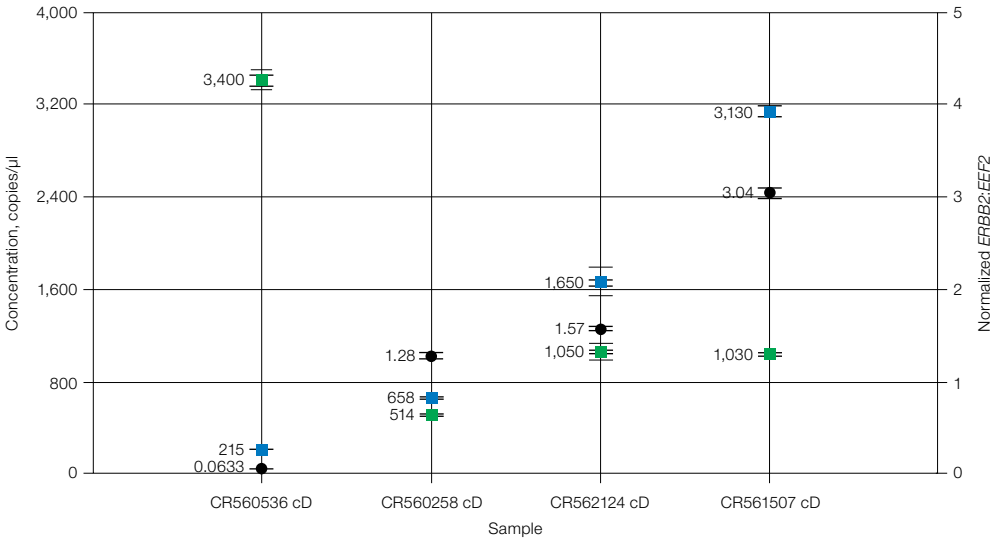

Fig. 6.3. Concentrations of *ERBB2* and *EEF2* gene expression.

Figure 6.4 shows a twofold change in sample concentration that demonstrates linearity of the assay spanning two log orders.

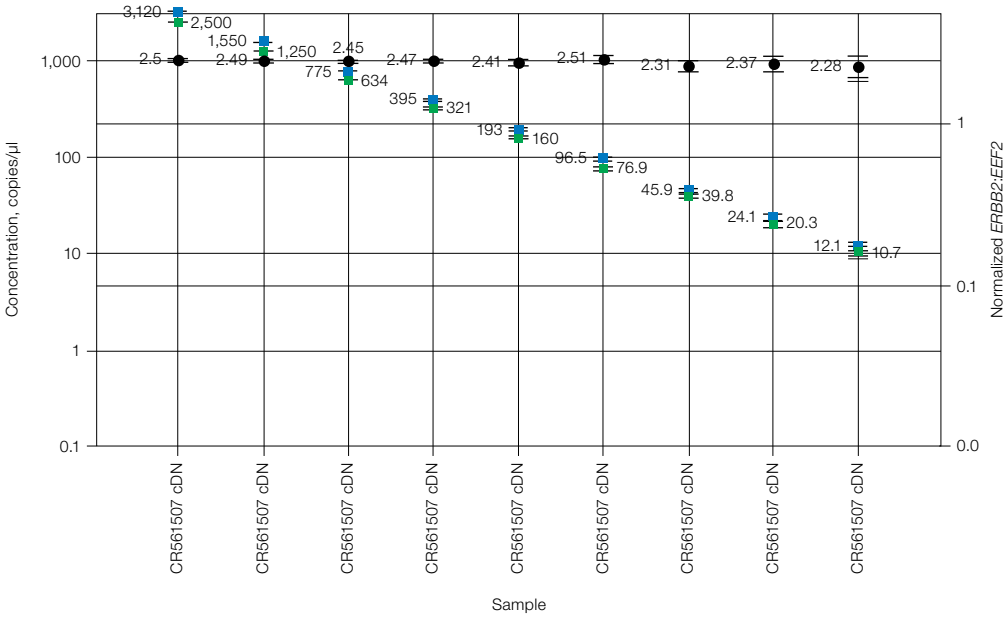

Fig. 6.4. Twofold dilution series demonstrating linearity across two log orders.

Figure 6.5 is an example of normal breast tissue compared with *HER2*+ tissues (fresh frozen).

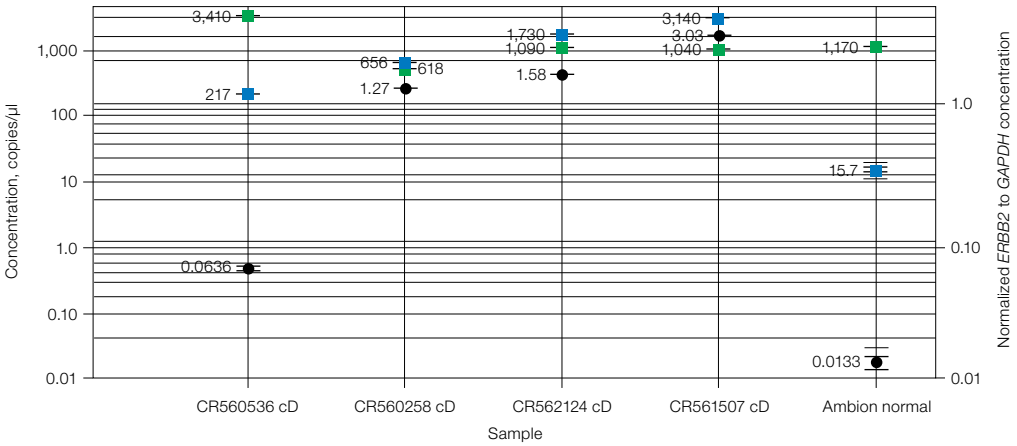

Fig. 6.5. Comparison of *HER2* expression levels of fresh frozen tumor versus normal tissue.

Figure 6.6 shows the quantification of RNA transcript levels compared with DNA copy number. Commercial (Applied Biosystems) *ERBB2*, *GAPDH*-PL, and *EEF2* gene expression TaqMan assays were tested with breast tumor FFPE samples. ddPCR was used to determine the amounts of transcripts in a subset of 12 samples (from the genomic copy number study above) that are normalized to commonly expressed genes *GAPDH* and *EEF2*.

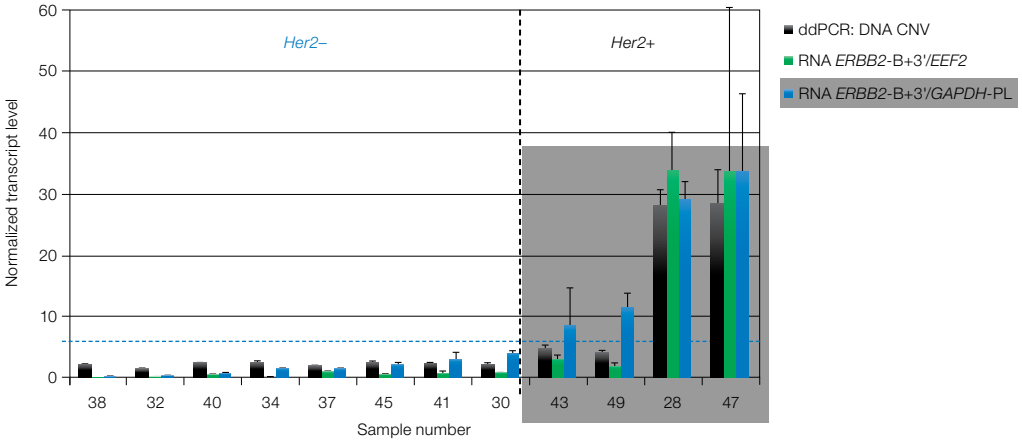

Fig. 6.6. Rank ordering of gene expression levels in 12 clinical samples and genomic copy number status of *ERBB2*.

When we studied the clinical FFPE samples using ddPCR and standard FISH and IHC methods, our results demonstrated that ddPCR can rank order the samples in complete agreement with the current standard methods and that ddPCR has the added benefit of providing quantitative results.

Table 6.1 shows a comparison of IHC, FISH, genomic copy number, and transcript level in clinical samples. The table shows the capability of ddPCR to determine gene copies and *ERBB2* cDNA transcript levels when normalized to a reference transcript (*EEF2* or *GAPDH)*. ddPCR results are in excellent agreement with pathology results, and have the added benefit of being quantitative.

Table 6.1. Comparison of clinical pathology results with ddPCR gene expression data.

		Clinical Pathology		ddPCR: CNVs or Normalized RNA Levels		
Sample Number	**Year Specimen Taken**	***HER2* IHC Score**	**FISH *HER2/CEP17* Ratio**	**DNA *HER2/CEP17***	**RNA *ERRB2-B+3'/EEF2***	**RNA *ERRB2-B+3'/GAPDH***
38	2009	1+	0.8	2.20	0.22	0.27
32	2010	1+	1.2	1.56	0.29	0.46
40	2008	2+	Negative	2.46	0.54	0.88
34	2009	1+	Not done	2.61	0.17	1.42
37	2008	0	Not done	1.96	0.98	1.54
45	2006	Negative	Not done	2.55	0.63	1.89
41	2008	1+ (resection)	Negative	2.53	0.85	3.01
30	2009	1+	Not done	2.37	0.86	3.87
43	2007	2+	Positive	4.76	2.97	8.31
49	2006	3+	Not done	4.15	1.79	11.3
28	2010	3+	Not done	28.0	33.9	29.1
47	2006	3+	Not done	28.4	33.6	33.7

CNV, copy number variation; FISH, fluorescence in situ hybridization; IHC, immunohistochemistry.

ddPCR provides the advantage of accurate and precise measurements of transcript levels. It also provides accurate genomic copy number determinations of samples with low- and high-order copy changes, determined by the ratio of the target concentration to the reference concentration. Important considerations for ddPCR applied to gene expression studies include the type of method used to obtain cDNA, one-step versus two-step reverse transcription, and optimizing assays for maximum performance.

7 Next-Generation Sequencing Library Analysis

Overview

Next-generation sequencing (NGS) systems are extremely sensitive to the quantity of library loaded in the sequencing run. Overloading frequently produces unusable data and underloading wastes reagents and time. Droplet Digital™ PCR (ddPCR™) complements NGS by offering accurate library concentration measurements and unique quality analyses that are not available with other methods.

Standard methods for quantifying NGS libraries have disadvantages. Electrophoresis and spectrophotometry measure total nucleic acid concentrations, whereas optimal cluster density or template-to-bead ratio depends on the appropriate concentration of PCR-amplifiable DNA molecules. Incorrectly adapted products or adapter-adapter dimers cannot be distinguished easily from bona fide library fragments. These undesirable species can compete for binding sites on the flow cell, inhibit cluster formation, increase the likelihood of suboptimal loading, and reduce the total number of high-quality reads. These methods also have low sensitivity, consuming nanograms of precious samples, and are not suitable for high-throughput workflows. A more accurate method to quantify libraries before sequencing is required to maximize usage of sequencing platforms.

ddPCR provides an absolute, standard-free method to measure library concentration and quality, while optimizing overall NGS performance. ddPCR makes very precise and accurate measurements of the library stock concentrations for flow-cell loading. The digital nature of droplet partitioning is conducive to accurate quantification by reducing competing PCR reactions.

ddPCR Quantification on Illumina TruSeq v2 Chemistry

For the Illumina MiSeq and HiSeq platforms, the total possible reads is directly related to the concentration of prepared library loaded. These platforms have a narrow loading concentration range requirement for successful runs. To maximize the sequencing information from a given sequencing run, accurate measurements of library concentration must be made.

Measuring concentration by ddPCR before amplification may help determine the number of additional PCR cycles needed, if any, to obtain enough library for loading.

Measuring concentration by ddPCR before the library is loaded for sequencing determines concentration and helps identify any library construction quality anomalies accumulated during the process.

The TruSeq v2 library preparation protocol is shown in Figure 7.1. TruSeq Y-adapters, containing both P5 and P7 sequences, are ligated to library DNA inserts. Following PCR amplification, the resulting amplicons contain P5 and P7 sequences directionally oriented on either strand of the fragment library.

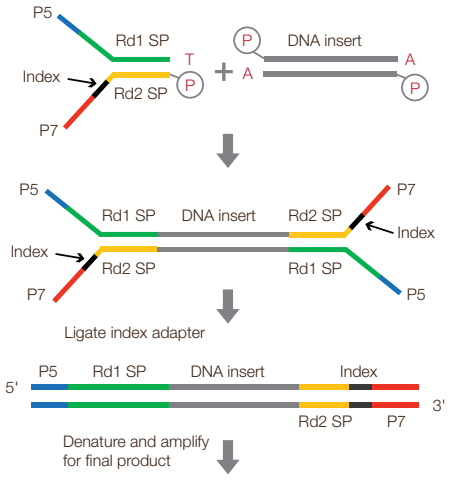

Fig. 7.1. TruSeq v2 library preparation.

Our duplex assays target the flanks of the library fragments with two probes targeting both the P5 and P7 moieties. We can directly measure linkage between probes 1 and 2 because they are co-localized in the same droplet (Figure 7.2). Targeting both flanking segments ensures testing for both well-formed and poorly formed fragments, allowing quantification of species possessing both adapter arms.

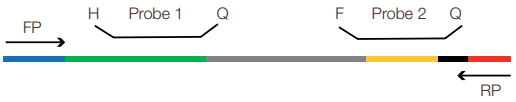

Fig. 7.2. Design of the ddPCR library quantification kit for Illumina TruSeq assay.

An example of the 2-D plot of ddPCR FAM versus HEX data observed for this assay is shown in Figure 7.3.

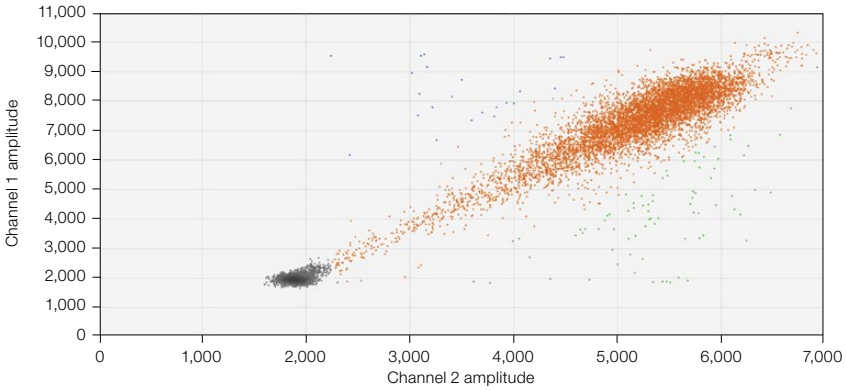

Fig. 7.3. 2-D plot of ddPCR data from the ddPCR library quantification kit for Illumina TruSeq.

Library Quality Analysis

The ddPCR library quantification kit for Illumina TruSeq contains TaqMan assays designed to detect and quantify both the P5 and P7 adapter arms. Each assay can be used to independently confirm the formation of bona fide library fragments by exploiting the ability of the QX100™ or QX200™ Droplet Digital PCR system to detect subpopulations of templates with differing amplification efficiencies and with different combinations of P5 and P7 moieties.

A 2-D plot of the library can be used to provide information on the quality of the library (Figure 7.4). Inserts of varying sizes within the library can be observed along a diagonal path, with larger fragments near the negative droplets and smaller inserts in the upper-right corner. This is because the smaller amplicons produce greater fluorescence during PCR amplification. This characteristic pattern enables you to assess the quality of your library. At the highest point in the upper-right corner is a population that appears separate from the bulk of the library. This population is the adapter-dimer population with no inserts (red circle in Figure 7.4).

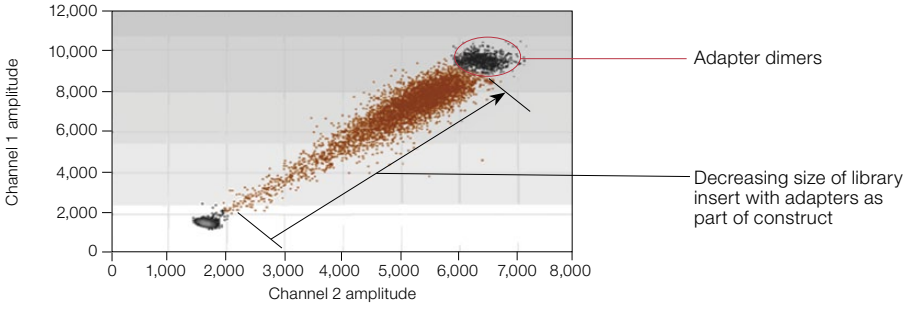

Fig. 7.4. Additional library information produced by ddPCR.

Droplets that appear above and below the insert population (large, diagonal cluster of increasing fluorescence) represent rare species with three or more adapters ligated to the insert (Figure 7.5). These populations can be selected in QuantaSoft™ software using the lasso function. By selecting the desirable bulk population that lies along the diagonal (green circle, Figure 7.5), excluding the adapter-adapter population in one color channel (blue circle, Figure 7.5), and excluding the undesirable side populations (adapter-adapter and extra adapter populations in red circles, Figure 7.5), you can then use the Ratio tab in QuantaSoft software to select **Fractional Abundance (a/a+b)**, and get a readout of the percentage of your library with inserts. This can be a quick and easy readout of the quality of your libraries. As anything with two adapters can still contribute to your cluster densities, you must include the adapter-adapter populations in your quantification and balancing of your libraries for sequencing. This quality measurement can be used to enable you to appropriately load and balance your sequencing runs, thus compensating for poorly formed library fragments and improving greater reading depth.

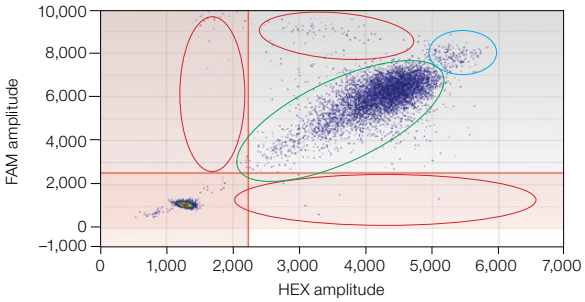

Fig. 7.5. Various species visualized by the ddPCR library quantification kit assay.

The library quality information obtained from 2-D plots will fuel further investigations into improvements of NGS sequencing, possibly by determining library fragment PCR efficiencies. Improvements in the NGS workflows will likely result from extremely accurate quantification by ddPCR. For example, you may be able to eliminate secondary amplification steps if enough library material is generated for sequencing directly after library construction, thus avoiding unnecessary steps and further skewing of fragment representation.

Next-Generation Sequencing Reads

In Figure 7.6, we demonstrate the relationship between the ddPCR-determined library concentration loaded and the number of total reads from sequencing on a MiSeq platform. By measuring library concentrations, the amount of input can be balanced across samples before loading the NGS instrument.

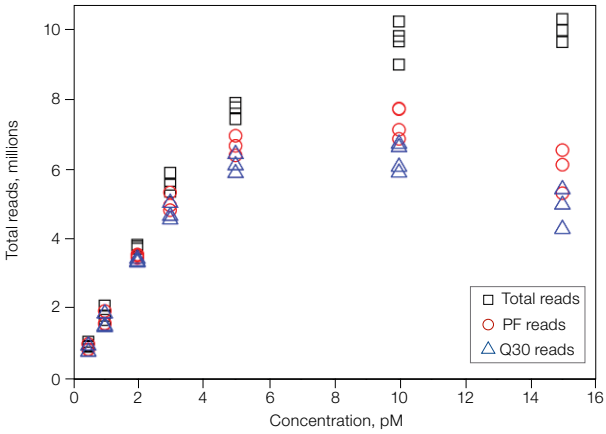

Fig. 7.6. Plot of the reads vs. input library concentration. Impact of input library concentration on total usable reads. Cluster density at 5 pM was approximately 800,000/mm^2. PF, passing filter.

As the cluster density and therefore number of reads is intimately tied to the loading concentration of the prepared library, moderate differences can compromise read capacity and quality of the MiSeq platform. NGS library quantification with ddPCR is extremely accurate, providing accuracy better than 15% with a confidence level of 95%. This method provides absolute quantification, eliminating the need to develop standards. Use of ddPCR in NGS significantly increases reliability and quality, and optimizes use of consumables, labor, and instrument time. Additional information, not available with other methods, such as adapter-adapter dimers and improperly adapted species can be seen. We have observed an inverse relationship between the size of the amplicons and fluorescence intensity with our ddPCR library quantification kit for Illumina TruSeq. The smaller the amplicon size, the higher the fluorescence attained, most likely due to PCR efficiency within the droplets. This information-rich content provides you with a digital quality check in the library construction before a sequencing run.

Library Balancing

When performing NGS on the MiSeq platform, it is important to aim for a cluster density of approximately 800,000/mm^2 for optimal performance. ddPCR measurements can be used to establish the functional relationship between input library concentration and the number of usable reads on the MiSeq platform.

We examined the precision of ddPCR in balancing 12 TruSeq DNA libraries from human genomic DNA using concentration measurements obtained from the QX100 system using the ddPCR library quantification kit (Figure 7.7). Based on the ddPCR concentration measurements, libraries with an average fragment length of 447 bp could be balanced within less than 15% of each other with a confidence interval of 95%. Similar balancing results were observed when RNA-Seq libraries with an average fragment length of 280 bp were used.

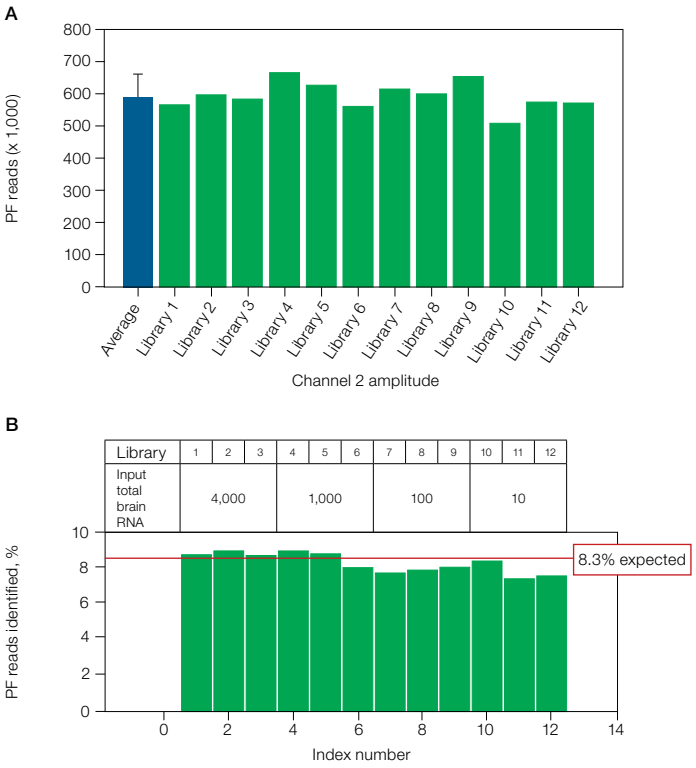

Fig. 7.7. ddPCR library balancing results. PF, passing filter.

Amplicon Recovery from Droplets

Bio-Rad's ddPCR supermix for probes (no dUTP) has been optimized for PCR amplification of rare target DNA sequences and for NGS library preparations. The droplet PCR supermix provides unbiased amplification and greater template coverage of sequencing template.

Generate droplets by following the ddPCR standard workflow and protocols. If your goal is to read droplets as well as recover material from droplets, make the desired number of wells to be read on the QX100 or QX200 system (nonrecoverable), and also make replicates to be broken open (not to be read on the QX100 or QX200 system).

For example, a column of wells could be generated (eight wells), four of which are read after PCR and four of which are not read. In QuantaSoft software, set up the plate where only four of the eight wells are read. After the QX100 or QX200 system has finished the run, remove the plate and pierce the foil of the four remaining unread wells and proceed with breaking the droplets from those wells.

If your goal is to generate droplets and break them open only after PCR, without reading them on the QX100 or QX200 system, then proceed directly with the following protocol for recovery of DNA from droplets after PCR:

1. Pipet out the entire volume of droplets and oil from a well into a 1.5 ml tube (combine up to ten replicates if desired).

2. Pipet and discard the bottom oil phase after droplets float to the top.

3. Add 20 µl of TE buffer for each well used; add additional TE buffer by multiplying by the number of combined replicate wells if applicable.
4. In a fume hood, add 70 µl of chloroform for each well and cap the tube. Add additional chloroform by multiplying by the number of combined replicate wells if applicable.
5. Vortex at maximum speed for 1 min.
6. In a centrifuge, spin down at 15,500 x g for 10 min.
7. Remove the upper aqueous phase by pipetting, avoiding the chloroform phase, and transfer it to a clean 1.5 ml tube (this is the recovered DNA).
8. Dispose of the chloroform phase appropriately.
9. If desired, estimate size using Bio-Rad's Experion™ DNA 1K analysis kit chip and/or requantify by ddPCR.

In Figure 7.8, the contents in the Eppendorf tubes following the droplet breaking protocol demonstrate the layers formed by chloroform and the broken aqueous layer. The upper aqueous phase can readily be pipetted into a clean tube for downstream analysis.

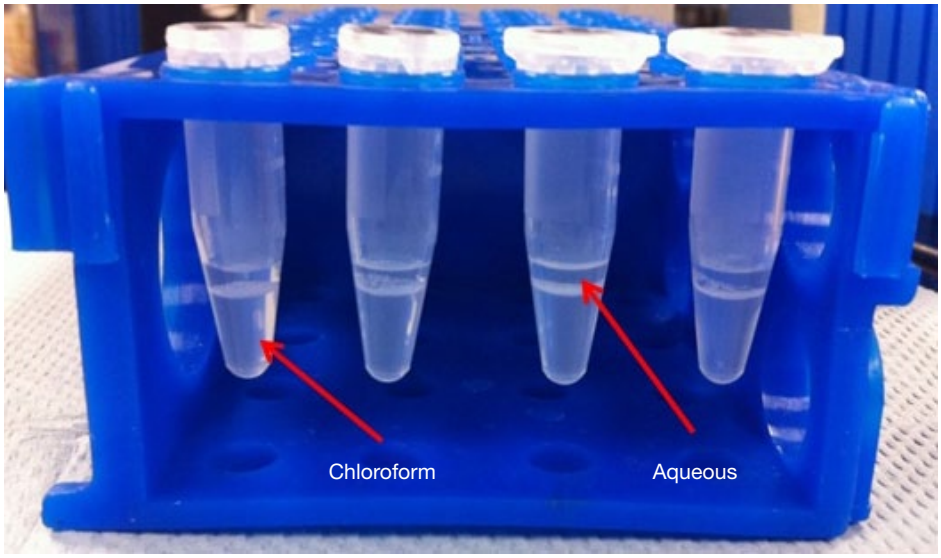

Fig. 7.8. Aqueous phase recovery following droplet amplicon recovery protocol.

Recovered DNA can be analyzed by gel electrophoresis (Figure 7.9), sequencing, and ddPCR (Figure 7.10).

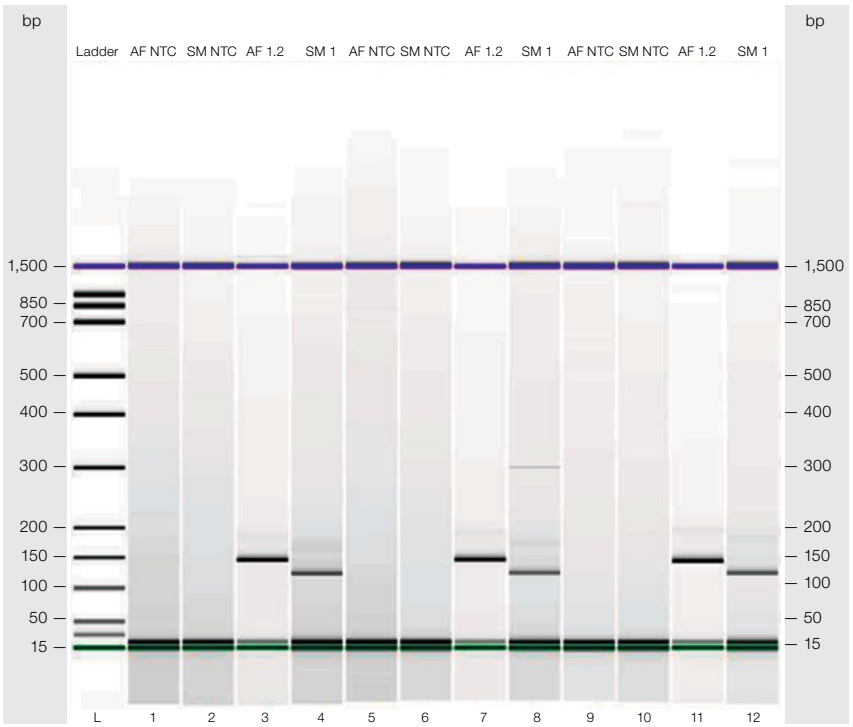

Fig. 7.9. Postrecovery analysis of ddPCR products. bp, base pair; NTC, no template control.

In Figure 7.9 four replicate wells are shown for each assay, of which three were combined for the droplet breaking protocol, and the fourth was read by the QX100 droplet reader. Gel analysis and electropherograms of amplicon products made from two different reactions prepared in droplets and no template controls are shown in the panels to the left. Lanes 1, 2, 5, 6, 9, and 10 are no template controls of the reactions; lanes 3, 7, and 11 are a *Staphylococcus aureus* assay; and lanes 4, 8, and 12 are a different assay for the *S. aureus* template.

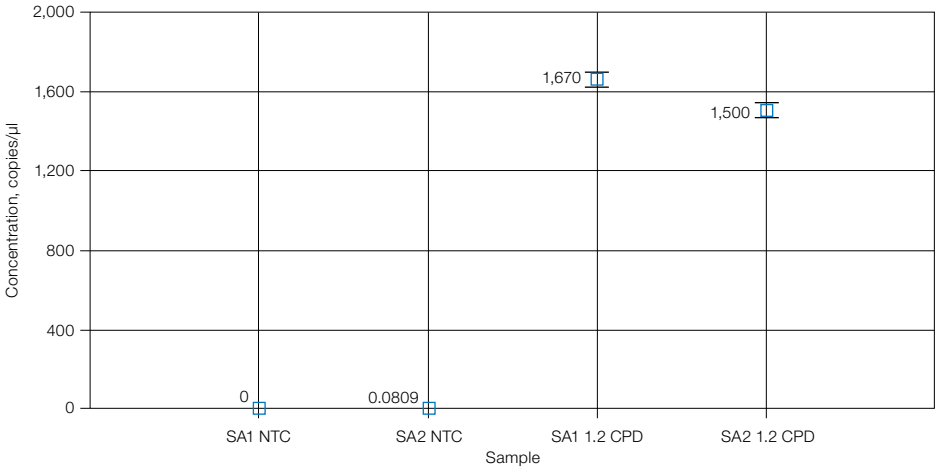

Fig. 7.10. ddPCR quantification of droplet recovered amplicons. CPD, copies per droplet; NTC, no template control; SA1, *S. aureus* assay 1; SA2, *S. aureus* assay 2.

Results of ddPCR concentration plots for the two *S. aureus* reactions carried out in the example are shown.

8 Additional Applications

Linkage Analysis

Physical linkage of two alleles in Droplet Digital™ PCR (ddPCR™) can be thought of in at least two ways:

- Tandem repeats of the same sequence
- Proximity of two sequences (targets) physically linked on the same piece of DNA

We recommend using restriction enzymes to digest the DNA in a copy number variation (CNV) study and physically separate the two target copies (in order to ensure random target distribution into droplets). If the copies are not separated, then the target can be undercounted because the copies will always segregate into the same droplet. This is especially true for tandem copies near each other. The extreme sensitivity of ddPCR, however, allows you to study linkage between targets and to infer the haplotype of samples by comparing digested vs. undigested DNA.

Figure 8.1 represents a study of different human samples using restriction digestion in comparison with undigested DNA for the same sample for the *MRGPRX1* gene, which has variable copy numbers (CNs) in different populations. In samples 4 and 5, the undigested (uncut) samples have lower CNs compared to the digested (cut) DNA of the same sample, which suggests the copies in those samples are linked on the same chromosomal strand.

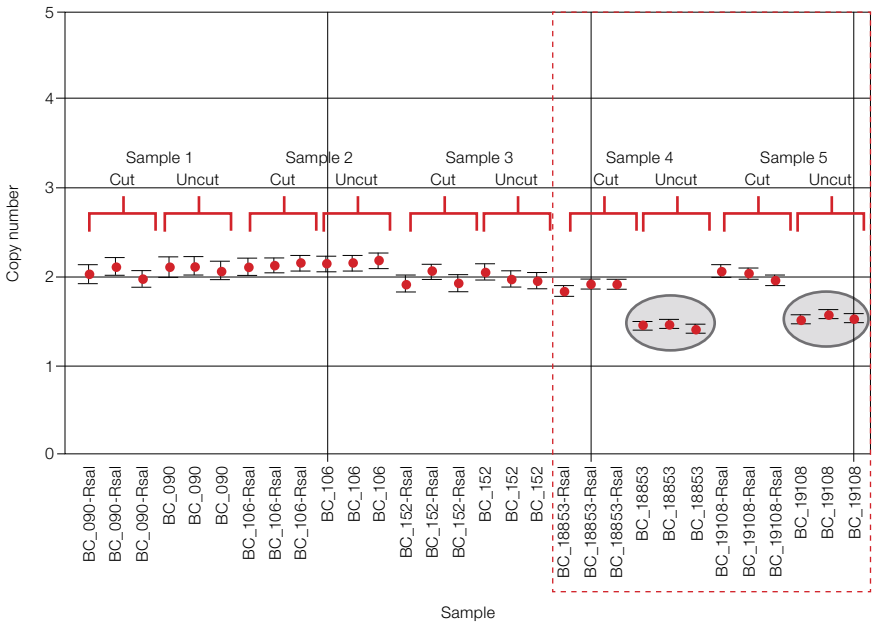

Fig. 8.1. CNV of different human samples using restriction digestion vs. undigested DNA for the same sample for the *MRGPRX1* gene.

Milepost Assay

Linkage studies can also provide information on the quality of the template, and allow you to determine how degraded or fragmented the template might be by examining an assay that serves as a positional anchor and a second assay located at a determined distance on the same chromosome. This is known as a milepost assay.

Figure 8.2 represents the assay design scheme for performing a milepost assay. Different DNA targets are designed at different lengths from an anchor assay, typically 1 kb, 10 kb, and 100 kb. Each milepost marker is run in multiplex with the anchor assay.

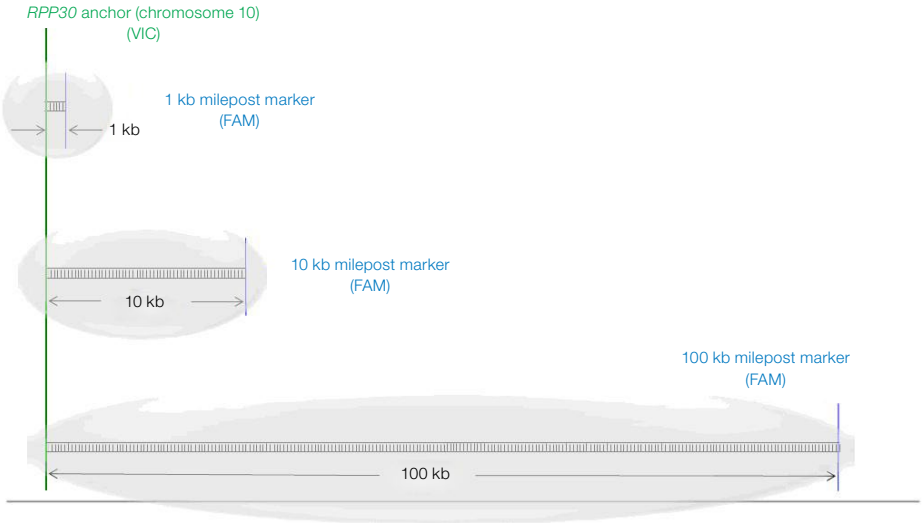

Fig. 8.2. Assay design for the milepost assay.

Demonstration of a milepost assay is presented in Figure 8.3, which shows the 2-D plots of FAM amplitude and VIC amplitude for an RNaseP anchor assay (VIC), and progressively farther assays on chromosome 10 for a human DNA sample. The upper left panel demonstrates a control to account for the inherent probability of two copies completely separated from each other (RNaseP, which is on chromosome 10, and an assay located on chromosome 6) randomly co-localizing in the same droplet. The remaining panels demonstrate that as the distance between the anchor and the milepost assay (on the same chromosome as RNaseP) increases, the number of double-positive droplets decreases. At a distance of 100 kb, the double-positive population is equal to the unlinked control. Finer resolution could be done with more milepost assays to cover the range.

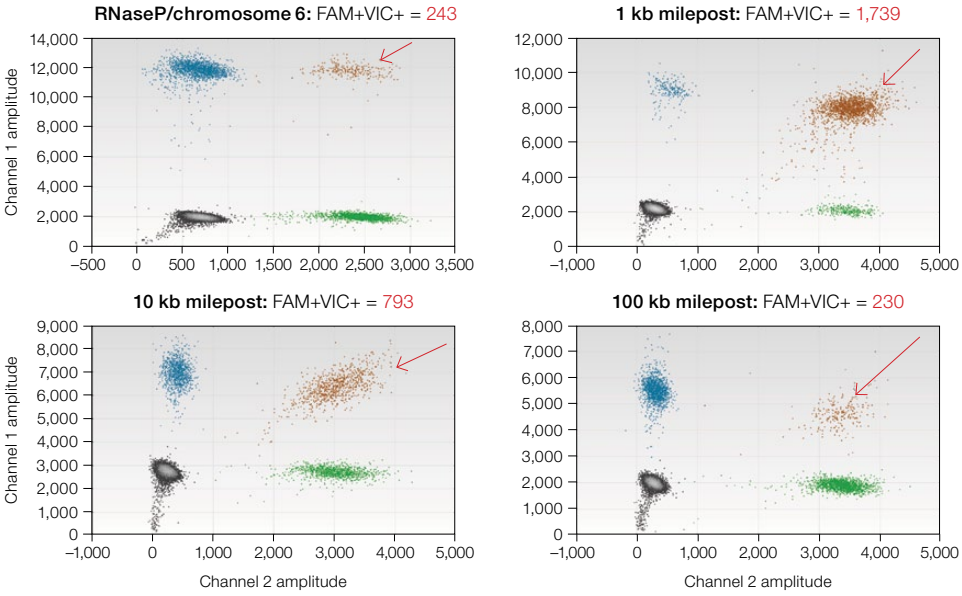

Fig. 8.3. Milepost assay results to determine the quality of DNA by a linkage study.

microRNA Amplification by ddPCR

A method for absolute quantification of microRNA (miRNA) described here shows the day-to-day reproducibility of a tenfold change in the starting quantity of synthetic template mir-210. An example ddPCR amplification of an miRNA synthetic template (mir-210) was done in a two-step reaction. The reverse transcription (RT) reaction was done in bulk solution, and the cDNA was partitioned into droplets before PCR amplification.

miRNA synthetic template for mir-210 (RNase free, HPLC purified) was obtained from Integrated DNA Technologies, Inc. The miRNA synthetic templates in TE buffer at a final concentration of 1 µM were aliquoted into individual-use tubes and frozen at –80°C. Each miRNA stock was loaded in RNase-free water on ice just before performing an RT assay. Reverse transcription of the synthetic miRNA templates was done in bulk solution using the TaqMan microRNA reverse transcription kit (Life Technologies Corporation), and using the miRNA RT protocol with the specific RT primers for each miRNA.

ddPCR amplification of the cDNA generated in the RT reaction was done by adding 1.33 µl of each cDNA to a 20 µl ddPCR reaction mixture containing 1 µl of 20x TaqMan miRNA reagent (Life Technologies) specific to the miRNA of interest, 10 µl of Bio-Rad's 2x ddPCR supermix for probes, and 7.67 µl of molecular biology–grade water. The droplets were generated, thermal cycled, and detected using standard procedures.

Day-to-Day Reproducibility Study: mir-210 miRNA

mir-210 synthetic templates at 1.6 nm originated from a single 1 µM stock. The 1.6 nM stock was used to create nine concentrations (10 µl into 90 µl RNase-free water) on ice. Concentrations 5–9 were used for the titration series, with an estimated concentration range of 2,135–0.21 copies/µl of ddPCR. The data are plotted as individual well replicates (duplicates) for each day with Poisson 95% confidence intervals in Figure 8.4.

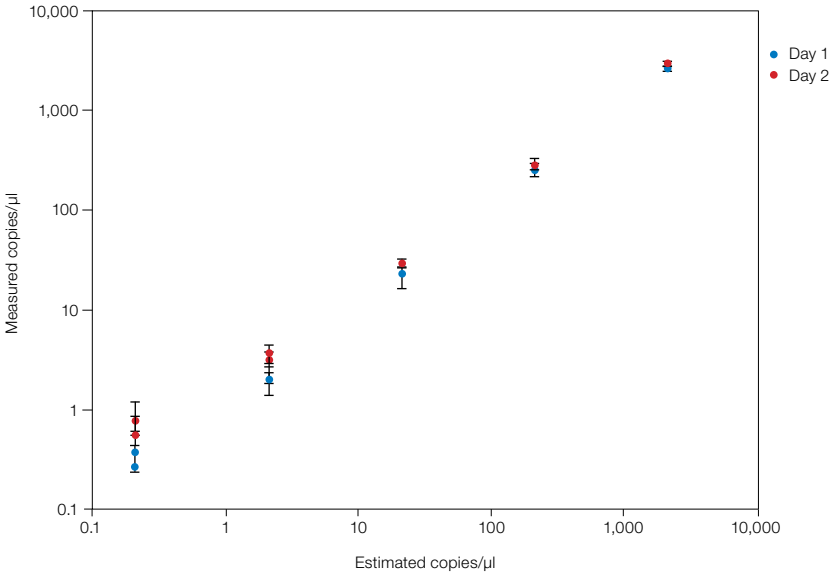

Fig. 8.4. Day-to-day reproducibility of mir-210 miRNA assay: measured vs. theoretical.

Multiplexing

Various schemes can be designed to add complexity to assays run in ddPCR. For instance, you may want to add more than two assays to a reaction. The QX100™ and QX200™ Droplet Digital PCR systems can detect fluorescence in two color channels (FAM and HEX).

However, there is an added dimension of fluorescence amplitude that can be used to multiplex more than two assays at a time.

The basic concept for multiplexing is to make use of the differences in fluorescence amplitude signal to change the spatial positioning of the droplet clusters in the 2-D data plots drawn in QuantaSoft™ software. By varying the concentration of the TaqMan assay, the end-point fluorescence amplitude can be shifted in many assays. Due to this shift, you can include two or more assays in the FAM and/or HEX channels and empirically determine what concentrations will resolve the two assays in separate clusters.

A good starting point would be to halve, or conversely, double the concentration of an assay relative to the standard final 1x concentration in order to spatially resolve the two assays in a 2-D plot of FAM and HEX amplitudes.

Figure 8.5 is an assay targeting chromosome 10 of the human genome. The stock has the highest fluorescence amplitude and subsequent twofold changes in starting concentration demonstrate resolvable droplet populations based on fluorescence amplitude down to one-quarter dilution.

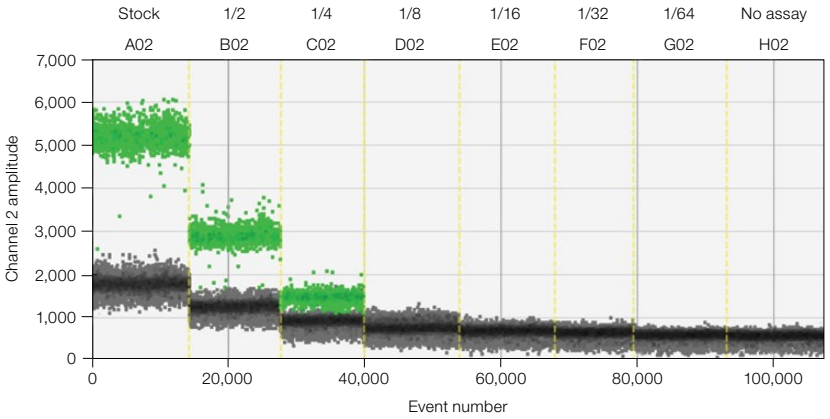

Fig. 8.5. Dilution series of an assay targeting chromosome 10 of the human genome.

Figure 8.6 represents an example of such a multiplexing strategy being used with one FAM channel assay and two HEX channel assays in a 2-D plot of the data.

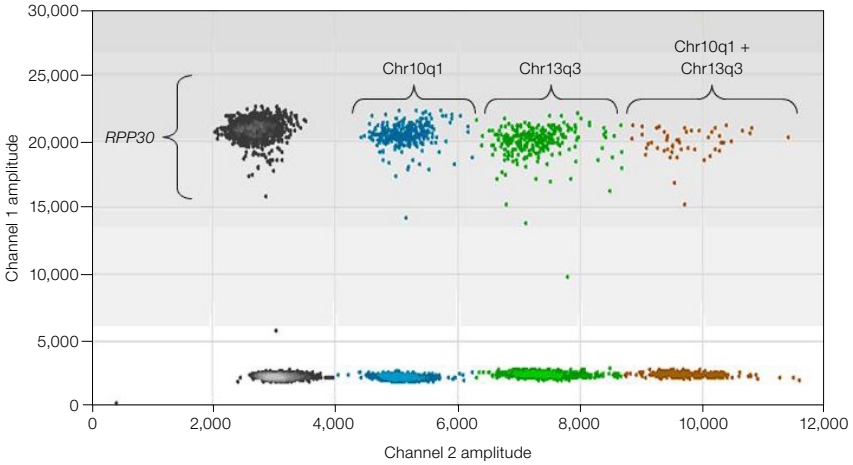

Fig. 8.6. 2-D plot of FAM amplitudes (channel 1) vs. HEX amplitudes (channel 2) for a multiplex experiment.

In this experiment, a single FAM assay was used (*RPP30*) at standard 1x final concentration and two different HEX assays at different concentrations (Chr13q3 and Chr10q1). Assay Chr10q1 was used at 1/2x standard concentration and assay Chr13q3 was used at 1x final concentration in the reaction. All three assays were present along with template in the supermix before making droplets. Using tools in QuantaSoft software to manually group populations, the four upper droplet clusters (gray, blue, green, orange) represent those droplets pertaining to droplets that are positive for the *RPP30* assay. The blue clusters are those droplets that are positive for the Chr10q1 assay. The green clusters are those droplets that are positive for the Ch13q3 assay. The orange clusters are those droplets that are positive for both Chr10q1 and Chr13q3 simultaneously. Negative droplets for the *RPP30* assay are the lower four droplet clusters (gray, blue, green, orange).

9 Droplet Digital™ PCR Tips, Assay Considerations, and Troubleshooting

Assay-Dependent Cluster Shifts

As with any PCR-based technology, assay design and sample preparation are important for obtaining good quality data. Before running a Droplet Digital PCR (ddPCR™) experiment, know the goal or possible expected outcomes of the experiment because different types of experiments require different controls, sample preparation, amounts of DNA or RNA, and data analysis.

Shifted Clusters Due to Probe Cross-Reactivity

If you see a shift inwards or upwards on the 2-D plot, this is most likely probe cross-reactivity. Probe cross-reactivity occurs when a probe binds to a nonperfect sequence and undergoes cleavage (Figure 9.1). This is more common in rare mutation detection (RMD) assays (for example, single nucleotide polymorphism [SNP] assays), where the two probes differ by only one base. The amount of nonspecific probe cleavage is a function of how close the melting temperature (T_m) of the mismatched probe is to the annealing/extension temperature used for the assay. For this reason, RMD assays are designed to maximize the T_m difference between a perfectly matched and a mismatched target. A cross-reacting probe causes single positive clusters (Ch1+/Ch2– and/or Ch1–/Ch2+) to migrate toward the axis of the other channel, which is most easily viewed in the 2-D amplitude plots (see Figure 9.1A). Figure 9.1 exemplifies this phenomenon where the FAM probe cross-reacts to a PCR product perfectly complemented by the VIC probe, resulting in mild to moderate FAM probe cleavage in the droplets. This causes an upward shift (or migration) in the location of the droplets in 2-D space. A similar shift is observed for the FAM+ droplets. These shifts are clearly seen in the 2-D amplitude plots. By contrast, the 1-D amplitude plots and histograms of these same data are difficult to interpret (Figures 9.1B–E). To avoid

such confusion, always classify the droplets of cross-reacting assays while viewing the 2-D amplitude plots. For users operating QuantaSoft™ software version 1.2.10 or earlier, appropriate droplet classification using a linear threshold may not be possible without misclassifying some droplets. Upgrading to QuantaSoft software version 1.3.2 or higher allows for proper classification using the clustering tools.

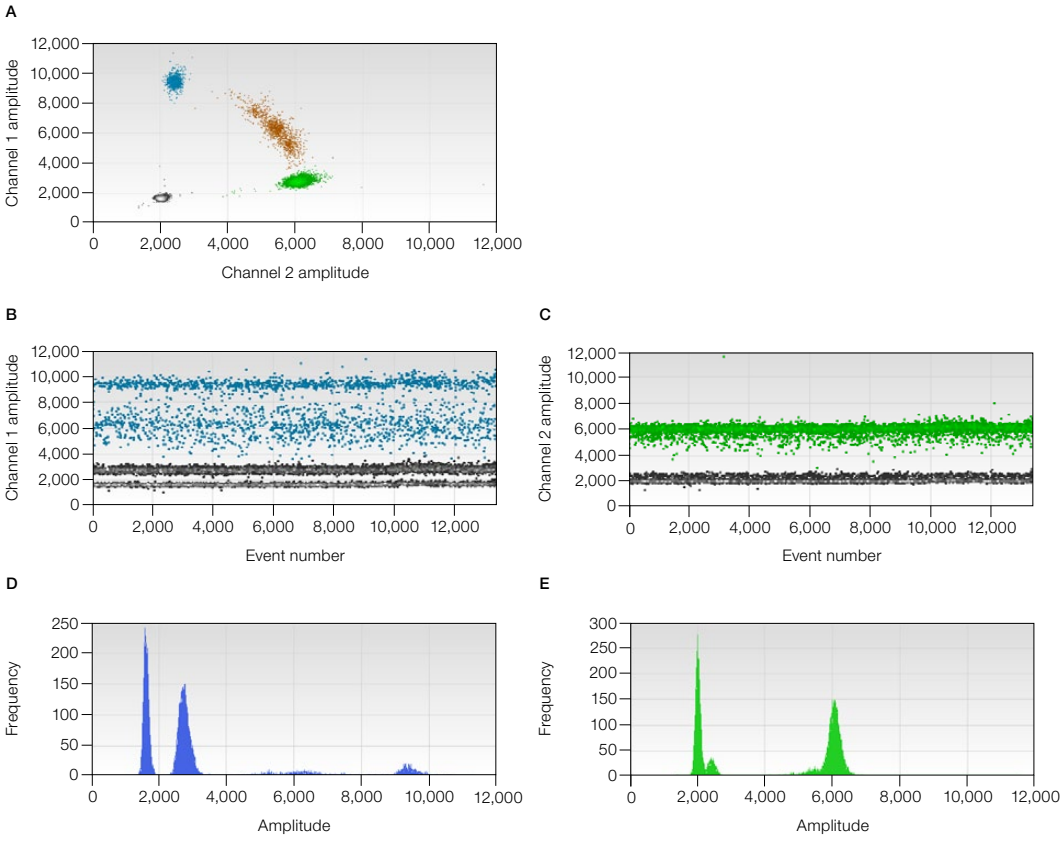

Fig. 9.1. Cross-reacting probes in a rare mutation detection assay. 2-D amplitude (**A**), 1-D amplitude (**B** and **C**), and histogram (**D** and **E**) plots.

Probe Cross-Reactivity Can Identify Off-Target Amplification

An unexpected extra cluster of positive droplets with fluorescence intensity less than the cluster containing the target of interest can be caused by a sequence variant in the target of interest (Figure 9.2). The droplets that cluster around 10,000 relative fluorescence units (RFU) contain a variant DNA sequence that is not perfectly matched to the designed probe. The perfectly matched sequence is the higher cluster around 12,000 RFU.

Often the distinction among two or more positive clusters is desirable because it provides additional information regarding the sample. If the mid-level cluster represents the detection of a potentially functional homolog, consider setting the threshold below this cluster to include it in the quantification, or lowering the annealing temperature so that these two clusters merge into one cluster. If the mid-level cluster is not desired, consider setting the threshold above this cluster to exclude it from target quantification.

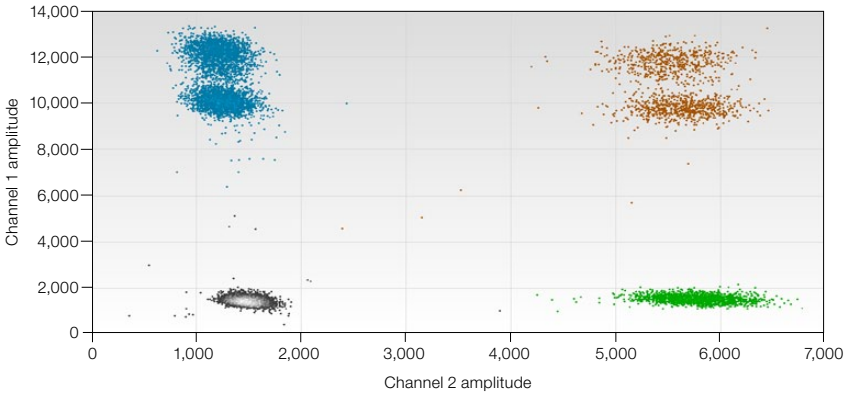

Fig. 9.2. Extra droplet clusters.

To prevent the off-target amplification, try increasing the annealing temperature of the PCR reaction to improve specificity (Figure 9.3), or alternatively, try digesting the sample with a restriction enzyme that will cut the nonspecific target while preserving the specific target. This latter approach requires some knowledge of the nonspecific target's sequence. Assay redesign can often be used to remove subclusters. Consider using Primer-BLAST to determine whether one of the primers is potentially nonspecific. If this is the case, examine whether a primer redesign would avoid the amplification of this product. If no flexibility exists in primer design, shift the probe to minimize complementarity to the off-target sequence.

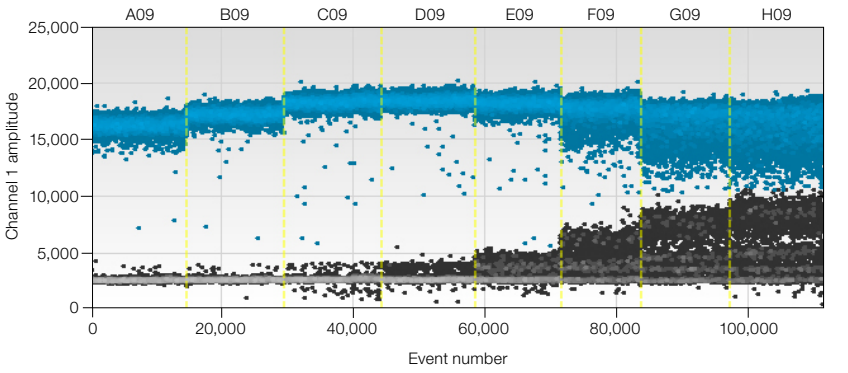

Fig. 9.3. Annealing/extension gradient where the assay detects an off-target species.

Positive Droplets in No Template Control Wells

Digital PCR can detect very low levels of target DNA so it is important to prevent template/amplicon contamination and to run no template controls (NTCs). Positive droplets in NTC wells that are at intensities equal to those of positive droplets in sample wells are typically caused by template or PCR product (amplicon) contamination in the reagents. Having a clean environment and clean NTC wells (that is, no positive droplets) is imperative when the application is rare sequence detection (wells with a low number of positives). In Figure 9.4, the first well (a contaminated NTC well) has four droplets of the same amplitude as those seen in the well on the right (positive sample).

If positive droplets in NTC wells occur, make sure that good laboratory practices for PCR are being followed in the laboratory (Kwok and Higuchi 1989).

Suggested guidelines are as follows:

- Wipe down pipets, tip boxes, and benchtops with 5–10% bleach
- Prepare master mixes in a template-free environment, add samples and generate droplets in an amplicon-free environment, perform PCR, and read droplets in a room separate from the sample preparations
- Do not reuse DG8™ droplet generator cartridges, oils, gaskets, plates, or pipet tips
- Wear appropriate personal protective equipment that is discarded or confined to appropriate locations (that is, template-free room for master mix assembly, amplicon-free room for template addition and droplet generation, and PCR and post-PCR rooms for droplet reading)

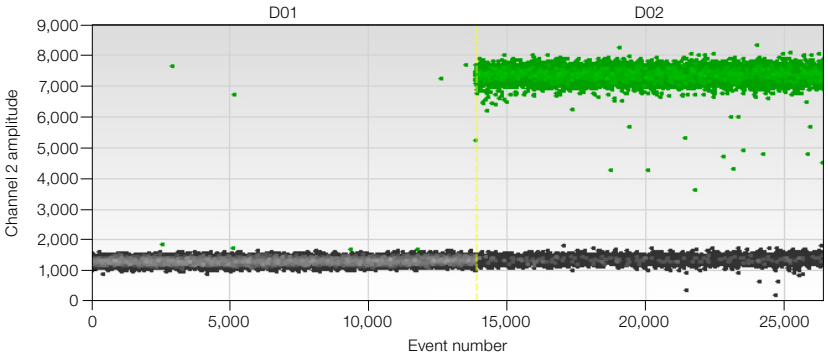

Fig. 9.4. An example of contamination of an NTC well (left) with amplitudes similar to a positive template reaction (right).

If desired, dUTP-containing supermixes (ddPCR supermix for probes) can be used in conjunction with heat-labile uracil N-glycosylase (UNG) or uracil DNA glycosylase (UDG) to reduce the potential for false positives resulting from the presence of previously amplified products. Add the UNG at 0.05 units/20 µl of ddPCR reaction mixture and create droplets as normal. Transfer the foil-sealed 96-well PCR plate containing droplets to the thermal cycler and add a 30 min 37°C incubation step in front of the standard recommended thermal cycling protocol. During this incubation period, UNG will digest U-containing products, such as those from past experiments performed with the ddPCR supermix for probes. UNG is heat inactivated during the first 3–10 min of the 95°C initial PCR hot-start step in the standard PCR protocol.

Note: The UNG approach can address contamination caused by PCR products created using ddPCR supermix for probes or one-step RT-ddPCR kit for probes; however, it will not address contamination caused by sample-source templates or PCR products created using droplet PCR supermix. Ultramers or long oligo PCR templates are especially problematic because the stock concentration is typically very high and can easily spread to pipets and other surfaces. It can be useful to order ultramers with uracils (Us) in place of a few of the thymidines (Ts) in order to take advantage of UNG treatment in case contamination becomes a problem.

High Mean Fluorescence Amplitude Intensity

If the fluorescence amplitude of negative droplets is excessively high such that they are all considered positive and therefore concentration cannot be determined, it is possible the sample's target concentration is so high that every droplet contains DNA target and no negative droplets exist (Figure 9.5). When there are no negative droplets, Poisson correction cannot be applied and it is not possible to calculate a concentration.

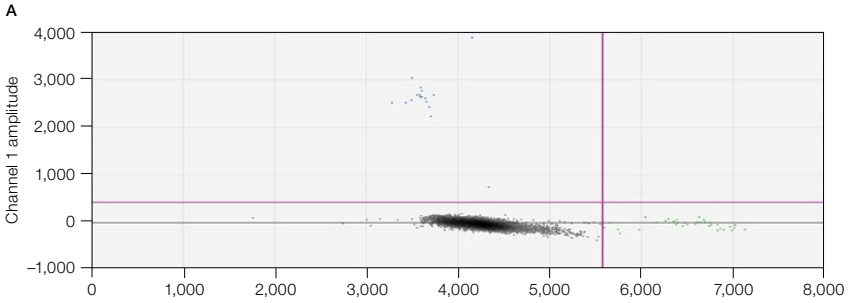

Fig. 9.5. Example of all droplets being positive (containing template) for the FAM assay and/or the VIC assay.

Having all positive droplets could arise from multiple issues.

1. Polymerase independent — probe hydrolysis due to poor long-term storage of probe stock solution, such as in a nonbuffered solution (for example, water) at 4°C.

   – Reorder the probe and make probe stock solution with 10 mM Tris, pH 8.0–8.5, and store at –20°C

2. Polymerase dependent — assay components interact with each other in a way that results in premature probe cleavage by the enzyme.

   – Identify intra-assay interactions and redesign causative component(s) to reduce binding and cleavage

Note: Run an NTC well to identify this problem. If the negative droplets in the NTC well do not have high fluorescence amplitude droplets, then the target concentration is too high. If the negative droplets in the NTC well contain high amplitude droplets, the cause is either polymerase-independent probe hydrolysis or polymerase-dependent intra-assay interactions.

No or Few Positive Droplets

If a new, never-before-tested assay fails to give positive droplets, consider the following:

1. The selected restriction enzyme may have cut within the target locus.

   – Recommendation: test the assay against DNA digested with a different restriction enzyme as well as undigested DNA

2. The target locus resides in a region that contains secondary structure.

   – Recommendation: use restriction enzymes to cut the sequences surrounding the region to be amplified in order to limit the number of possible interactions with nearby nucleotides

3. The assay does not work at the predicted temperature.

   – Recommendation: first perform an annealing/extension temperature gradient to determine the temperature at which the assay works

4. The ddPCR reaction mix was not assembled correctly or the probe/primers were not ordered correctly.

5. One of the assay components was designed incorrectly or a mistake was made during synthesis.

No or Low Total Droplet Count

To determine your droplet count, select the well in setup, click **Analyze**, then click the **Events** tab and make sure **Total** is selected. If the total accepted events or droplet counts are less than 10,000 consider the following recommendations:

- Use the recommended concentration of primer (900 nM), probe (250 nM), and 1x master mix. The QX100™ and QX200™ Droplet Digital PCR systems are compatible only with Bio-Rad's ddPCR supermixes. Using less than the recommended concentration of any of these components may lower your droplet count

- Load the DG8 cartridge with the appropriate volumes of sample and droplet generation oil (20 µl and 70 µl, respectively). If less than 20 µl of sample is loaded, fewer droplets will be generated. Be sure to load the sample before the oil

- Use only purified nucleic acids. Any particulate matter (for example, residual fibers from sample preparation columns or beads) in the sample should be removed before assembling the ddPCR reaction mixture because these particulates can clog the DG8 cartridge's microfluidic channels and disrupt droplet generation. To remove particulates from purified nucleic acids, spin the sample at 10,000 x g for 1 min and transfer the supernatant to a clean tube

- Do not exceed the recommended DNA load (66 ng/well undigested DNA or 1,500 ng/well digested DNA)
- Use only approved plates (Eppendorf twin.tec semi-skirted 96-well plates, catalog #951020362) with approved pierceable foil heat seals (Bio-Rad catalog #181-4040)
- Properly seal the 96-well plate. Under- or over-sealed plates result in oil evaporation during thermal cycling and compromise droplet data quality. If using the PX1™ PCR plate sealer (Bio-Rad catalog #181–4000), seal plates at 180°C for 5 sec. Do not use the PX1 sealing protocol twice on the same plate because this often disrupts the original seal
- Ensure that the full volume of the generated droplets is transferred into the 96-well plate by inspecting the DG8 cartridge after transfer
- Use only approved pipet tips for droplet generation and droplet transfer. Rainin and Eppendorf tips are approved for use

For sample loading, use P-20 pipet tips and slowly dispense the sample into the bottom of the DG8 well rather than pipetting at the top edge of the well. Then dispense 70 µl of oil into the oil wells. Begin droplet generation within 2 min of oil loading.

Use a manual P-50 pipet with a normal bore P-200 tip (not wide or narrow bore) to transfer droplets. Angle the P-200 tip in the well to prevent the droplets from having to squeeze between the pipet tip and well bottom (angle the tip position such that it is not vertical in the well). Slowly draw 40 µl of droplets into the pipet tip over ~5 sec. Typically ~5 µl of air will be pulled into the tip, which helps prevent the oil from leaking out.

Position the pipet tip (containing the droplets) near the bottom of the well and dispense the sample, ensuring ample room between the well and the pipet tip so that the droplets do not shear upon dispensing.

Inconsistent Concentration Results

Technical replicates of the same sample should yield concentration estimates that are within the Poisson confidence error bars 95% of the time. If the concentration estimates between technical replicates are not close, the most common causes are poorly mixed reaction mixtures or poor thermal cycler temperature uniformity.

Insufficient Mixing

When creating technical replicates, thoroughly mix the reaction mixture (master mix, sample, and assay) by pipetting the reaction mixture up and down ten times, using 90% volume strokes. Alternatively, pulse vortex the reaction mixture for 15 sec followed by spinning the sample down. Do not assemble or mix reaction mixtures in the DG8 cartridge. Figure 9.6 shows replicates that were not sufficiently mixed before droplet generation.

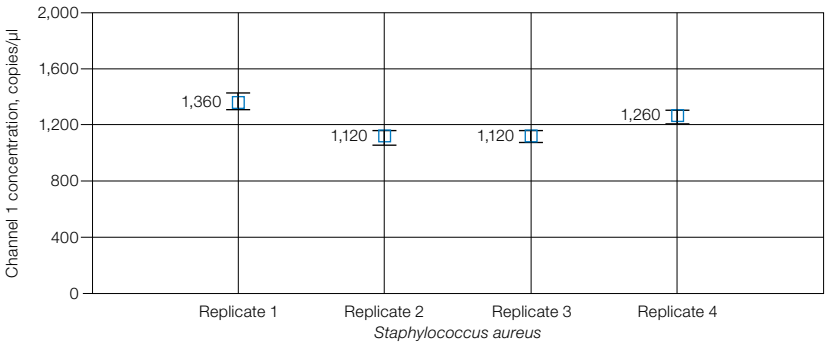

Fig. 9.6. Quadruplicate replicates drawn from the same poorly mixed reaction solution demonstrate inconsistent concentration readings.

Effects of Poor Cycler Uniformity

If the reaction mixtures used to create technical replicates are thoroughly mixed but there is wide variation in concentration estimates, consider the uniformity performance of your thermal cycler. Generally, this effect is observed only when a temperature-sensitive assay is used on a thermal cycler with poor uniformity. Uniformity at both the denaturation and annealing/extension temperatures is important. Bio-Rad's C1000 Touch™ thermal cycler with 96–deep well reaction module has excellent thermal uniformity. To test the module's uniformity, use the temperature-sensitive assay that has concentration variability and create droplets from the same reaction mixture for the entire plate. Check the entire plate for a discrepancy in concentration that exceeds the 95% confidence bounds for the wells. If one of the block's Peltier devices is broken or underperforming, a drop in concentration will be consistently observed in the same quadrant(s) of the block (Figure 9.7).

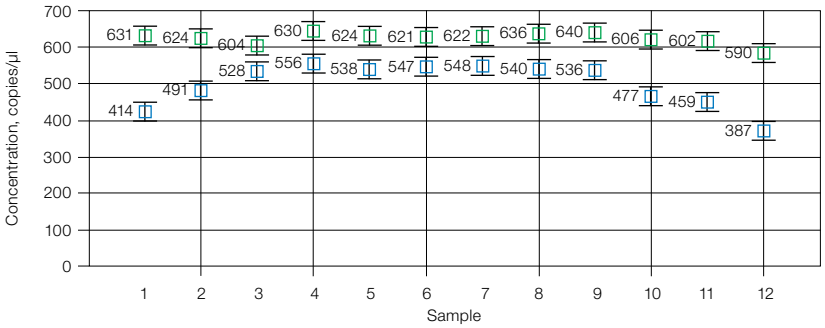

Fig. 9.7. Concentration plot of a temperature-sensitive assay (■) with a temperature-insensitive assay (■) concentration across the plate, despite having the same amount of input DNA/well.
All sample wells were loaded with 0.5 copies/droplet Raji DNA.

The temperature-sensitive assay used to generate Figure 9.7 is relatively long and very GC-rich (244 bp, 74% GC). If it is suspected that the variation in concentration is due to thermal cycler performance, consider:

- Increasing the hot start from 94°C for 10 min to 96°C for 10 min
- Raising the denaturation temperature from 94 to 96°C for the first 5 cycles
- Purchasing Bio-Rad's C1000 Touch thermal cycler with 96–deep well reaction module
- If drops in concentration estimates are consistently confined only to a quadrant(s) of the block, contact the manufacturer and request thermal-couple uniformity analysis and, if necessary, repair

Concentrations Consistently Lower than Predicted

If concentrations measured in ddPCR are consistently lower than predicted, consider poor target accessibility, poor or incorrect assay design, or the presence of PCR inhibitors in samples.

It is possible that the reference concentration measurement that suggests ddPCR concentration calls are low is, in fact, in error and is reporting a higher than actual concentration. ddPCR gives a concentration measurement of intact DNA targets while spectroscopic measurements typically do not distinguish between degraded and intact nucleic acids.

Also consider the following options:

- Make sure the ddPCR assay has been optimized by running a temperature gradient experiment
- Amplicons longer than 150 nucleotides may require longer annealing times during PCR
- If duplexing 2 assays together for the first time, test them in a singleplex assay using the same sample to confirm that the assays are not interfering with one another
- Verify that the fluorophore is not conjugated to a G residue
- Add the recommended primer (900 nM) and probe (250 nM) concentration

Additional Tips

No Concentration Calls on Some Wells

If a concentration estimate fails to appear in the concentration chart in QuantaSoft, this indicates the software could not auto-analyze or assign droplets to positive or negative populations using its auto-analysis algorithm, or the well had an unusually low droplet count (<10,000). Low total droplet counts indicate a problem with the assembly of the reaction mix, poor preparation of the sample, or poor handling. Manually set a threshold and QuantaSoft software will calculate a concentration, which will appear in the concentration chart.

Target Accessibility

Strong or excessive secondary structure can prevent a DNA target from being amplified. Human gDNA and plasmid DNA can usually be restriction digested to remove inhibiting secondary structure, thereby rescuing detection. RNA secondary structure is best addressed by changing the location of the assay, if possible, or reverse transcribing the assay at a warmer temperature.

Figure 9.8 is an example of poor target accessibility, manifested by the significant number of mid-level amplitude droplets (that is, rain), which is resolved by performing a restriction digestion on the DNA before ddPCR.

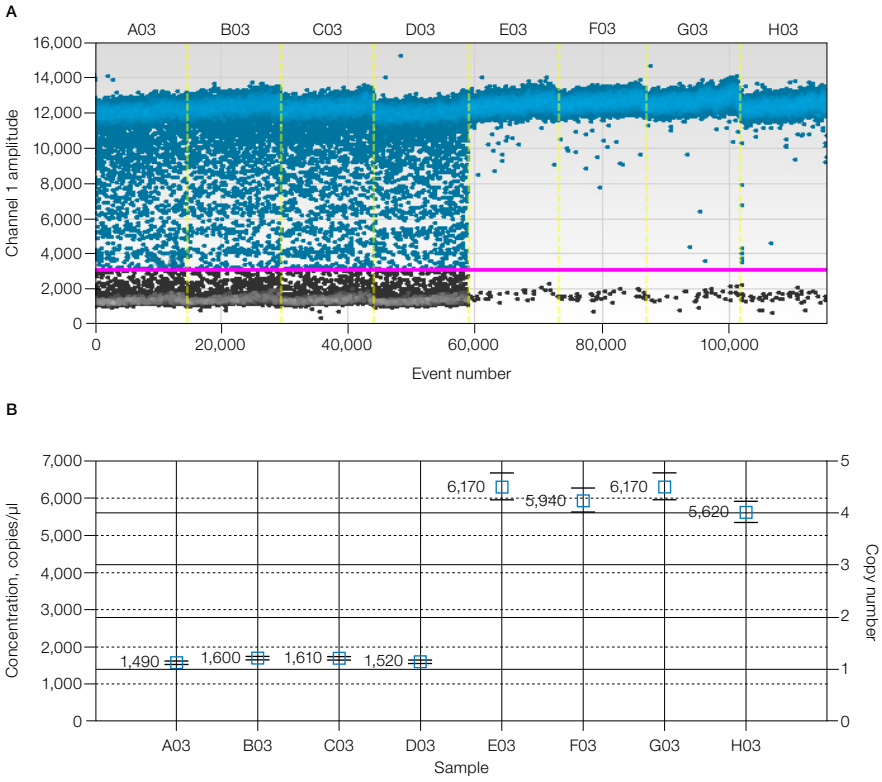

Fig. 9.8. Elimination of secondary structure allows efficient amplification and accurate quantification. **A**, fluorescence amplitude plot showing four wells of undigested plasmid DNA (left) and four wells after restriction enzyme digestion to linearize the plasmid (right); **B**, concentrations are corrected to the expected value after restriction enzyme digestion (right) as compared to undigested samples (left).

High-Fluorescence Amplitude Droplets

Droplet coalescence can create droplets that are much higher in fluorescence amplitude than the other positive droplets. This can be caused by poor droplet transfer technique or extended storage of the droplets pre– or post–thermal cycling. Adjust the scale on the 1-D or 2-D amplitude charts in order to set the thresholds in these cases.

Troubleshooting EvaGreen ddPCR Reactions

EvaGreen binds to both double-stranded DNA and, to a lesser extent, nonspecifically to single-stranded DNA. Therefore, adding excessive amounts of primer or DNA starting materials to EvaGreen ddPCR reactions can result in an inability to distinguish positive droplets from negative droplets. The separation of positive and negative fluorescence can be affected by total added DNA, including primer concentrations, in addition to the previously discussed fluorescence amplitude differences in amplicon length or amplicons with varying PCR efficiencies.

For good separation, a combined primer concentration between 75–250 nM is recommended and 100–200 nM is optimal (Figures 9.9A–B). Figure 9.9C is an example of the effect of EvaGreen ddPCR with 900 nM primers on increasing concentrations of DNA that should be negative for the primer set added. Figure 9.9D shows the primer concentration is decreased to 100 nM and the negatives are returned to a uniform cluster of droplets. Similarly, adding more than 130 ng DNA/20 µl reaction can cause the negative droplets and positive droplets to have poor separation. Figure 9.10 shows an experiment with 100 copies/µl mutant DNA spiked into increasing amounts of wild-type DNA. As the total added DNA concentration increases, the positive fluorescence amplitudes decrease and the negative fluorescence amplitudes increase.

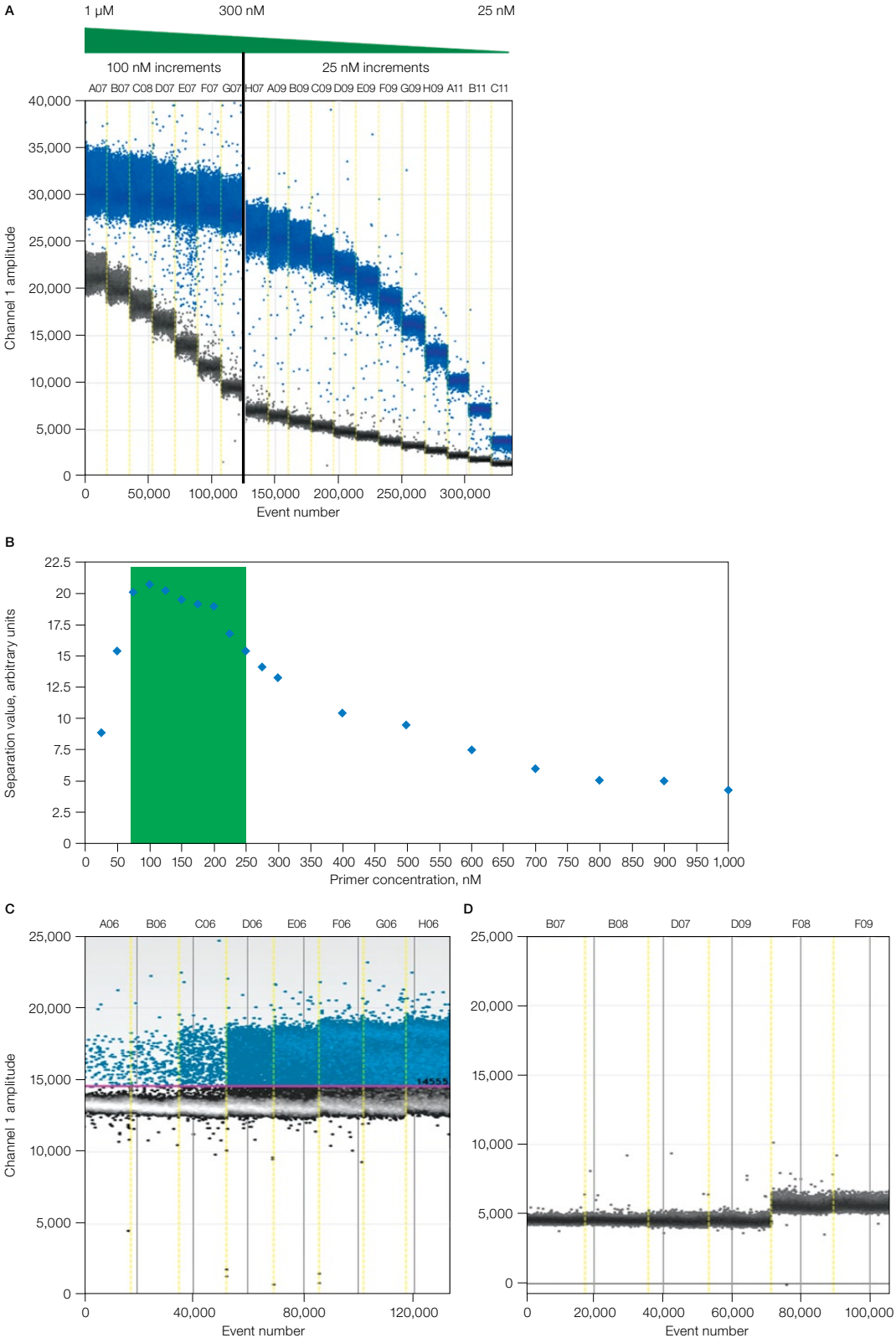

Fig. 9.9. Good separation of positive and negative droplets in EvaGreen ddPCR reactions. A–B, combined primer concentration between 75–250 nM is shown, with 100–200 nM being optimal; **C**, effect of EvaGreen ddPCR reaction with 900 nM primers on increasing concentrations of DNA; **D**, decreasing the primer concentration to 100 nM produces a uniform cluster of negative droplets.

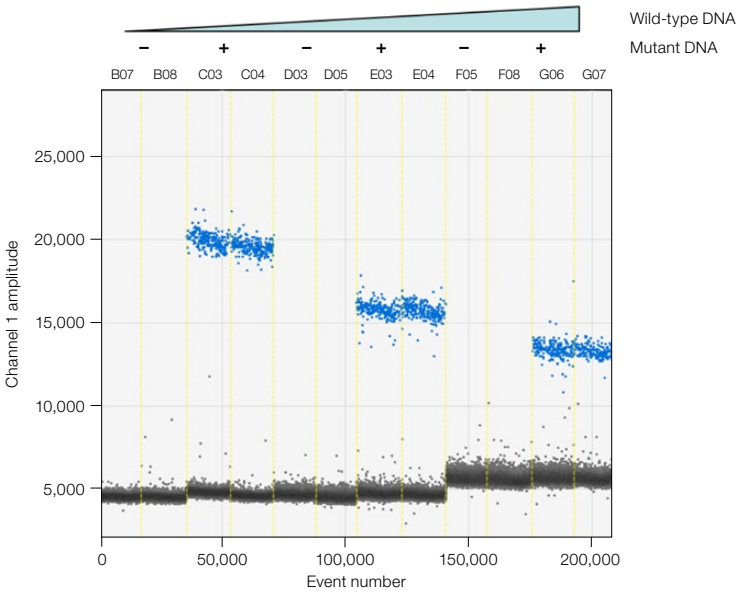

Fig. 9.10. Mutant DNA spiked into increasing amounts of wild-type DNA. As the total added DNA concentration increases, the positive fluorescence amplitudes decrease and the negative fluorescence amplitudes increase.

Reference

Kwok S and Higuchi R (1989). Avoiding false positives with PCR. Nature 339, 237–238.

Appendix A
Ordering Information

QX200™ Droplet Digital™ PCR (ddPCR™) System

186-4001 **QX200 Droplet Digital PCR System**, includes droplet generator, droplet reader, laptop computer, software, associated component consumables

186-4002 **QX200 Droplet Generator**, includes droplet generator, 1 pkg of 24 DG8™ cartridges, 1 pkg of 24 DG8 gaskets, 2 cartridge holders, 1 power cord

186-4003 **QX200 Droplet Reader**, includes droplet reader, ddPCR manual, 2 plate holders, USB cable, power cord

186-4007 **Droplet Generator Cartridges and Gaskets**, includes 5 pkg of 24 DG8 cartridges, 5 pkg of 24 DG8 gaskets

186-4008 **DG8 Cartridges for QX100™/QX200 Droplet Generator**, 1 pkg of 24 cartridges

186-3009 **DG8 Gaskets for QX100/QX200 Droplet Generator**, 1 pkg of 24 gaskets

297-11024 **Droplet Reader Plate Holder**

186-3051 **DG8 Cartridge Holder**

186-3005 **Droplet Generation Oil for Probes**, 10 x 7 ml

186-4005 **Droplet Generation Oil for EvaGreen**, 2 x 7 ml

186-4006 **Droplet Generation Oil for EvaGreen**, 10 x 7 ml

186-3004 **ddPCR Droplet Reader Oil**, 2 x 1 L

ddPCR Reagents

186-3026 **ddPCR Supermix for Probes**, 2 ml (2 x 1 ml), 200 x 20 µl reactions, 2x supermix

186-3010 **ddPCR Supermix for Probes**, 5 ml (5 x 1 ml), 500 x 20 µl reactions, 2x supermix

186-3027 **ddPCR Supermix for Probes**, 25 ml (5 x 5 ml), 2,500 x 20 µl reactions, 2x supermix

186-3028 **ddPCR Supermix for Probes**, 50 ml (10 x 5 ml), 5,000 x 20 µl reactions, 2x supermix

186-3023 **ddPCR Supermix for Probes (no dUTP)**, 2 ml (2 x 1 ml), 200 x 20 µl reactions, 2x supermix

186-3024 **ddPCR Supermix for Probes (no dUTP)**, 5 ml (5 x 1 ml), 500 x 20 µl reactions, 2x supermix

186-3025 **ddPCR Supermix for Probes (no dUTP)**, 25 ml (5 x 5 ml), 2,500 x 20 µl reactions, 2x supermix

186-3021 **One-Step RT-ddPCR Kit for Probes**, 2 ml (2 x 1 ml), 200 x 20 µl reactions, 2x RT-ddPCR mix, includes 1 manganese acetate tube

186-3022 **One-Step RT-ddPCR Kit for Probes**, 5 ml (5 x 1 ml), 500 x 20 µl reactions, 2x RT-ddPCR mix, includes 2 manganese acetate tubes

186-4033 **QX200 ddPCR EvaGreen Supermix**, 2 ml (2 x 1 ml), 200 x 20 µl reactions, 2x supermix

186-4034 **QX200 ddPCR EvaGreen Supermix**, 5 ml (5 x 1 ml), 500 x 20 µl reactions, 2x supermix

186-4035 **QX200 ddPCR EvaGreen Supermix**, 25 ml (5 x 5 ml), 2,500 x 20 µl reactions, 2x supermix

186-4036 **QX200 ddPCR EvaGreen Supermix**, 50 ml (10 x 5 ml), 5,000 x 20 µl reactions, 2x supermix

186-3052 **ddPCR Buffer Control Kit**, 9 ml (2 x 4.5 ml), 2x buffer

186-4052 **QX200 Buffer Control Kit for EvaGreen**, 9 ml (2 x 4.5 ml), 2x buffer

186-3040 **ddPCR Library Quantification Kit for Illumina TruSeq**, 200 x 20 µl reactions, includes ddPCR supermix for probes (no dUTP), (2 x 1 ml vials), ddPCR library quantification assay (1 x 200 µl vial), for quantification of Illumina TruSeq libraries using the QX100 or QX200 system

186-3041 **ddPCR Library Quantification Kit for Ion Torrent**, 200 x 20 µl reactions, includes ddPCR supermix for probes (no dUTP) (2 x 1 ml vials), ddPCR library quantification assay (1 x 200 µl vial), for quantification of Ion Torrent libraries using the QX100 or QX200 system

Thermal Cycler and Plate Sealer

185-1197 **C1000 Touch™ Thermal Cycler with 96–Deep Well Reaction Module**, includes C1000 Touch thermal cycler chassis, 96-deep well reaction module, USB flash drive

181-4000 **PX1™ PCR Plate Sealer**, includes heat sealing instrument, plate support block that holds 96-well and 384-well plates, sealing frame, power cord

Appendix B
Technical Error Bars in Droplet Digital™ PCR

Because Droplet Digital PCR (ddPCR™) is a digital counting system, you can calculate the measurement error based on the droplet data from a single well. This is the technical error (also referred to as the Poisson error in QuantaSoft™ software), and it is a good estimate of the errors you can expect to see on true technical replicates (defined as aliquots of the same starting material loaded into multiple ddPCR wells).

The technical error can be calculated from the data points obtained from a single well or multiple wells merged together. For a good assay, the technical error will be approximately equal to the standard error of the mean.

Conceptually, there are two contributions to the error bars: subsampling and partitioning.

Subsampling

In most molecular biology experiments, we analyze part of a whole (a subsample). Examples include:

- Blood sample
- Tumor biopsy
- Aliquot from a tube of DNA

Whenever you subsample from a larger volume with the intent to measure properties of the whole volume, random effects will lead to slightly different measurements from the subsampled volume. Subsampling error is most significant at low concentrations. While some quantification systems do not directly report the subsampling error, the standard error of the mean (the typical error reported for replicates) implicitly combines all the different sources of error, including the subsampling error.

Subsampling example: Suppose a person has a total of 100,000 copies of a particular target in his or her blood (5 L total volume) and you take 5 ml of plasma. On average, this 5 ml will contain 100 copies of target. But if you take 100 x 5 ml samples, about 16% of them will contain less than 90 copies and about 16% of them will contain more than 110 copies of target. This type of variability is inherent in any type of subsampling.

Figure 1 illustrates subsampling error that arises because a small volume was taken from a large amount of starting material. If the 12 µl of sample has 5 target molecules/µl and you take 1 µl of the sample (1/12th of the whole) and count the number of molecules in that subsample, you may expect to see 5 molecules. However, it is unlikely that you'll see exactly 5. Instead, you might see 3, 4, 6, 7, or 8 molecules. This uncertainty is what we mean by the subsampling error.

Subsampling statistics provide a lower limit on the measurement error, completely independent of the instrument used for measurement. Any additional error introduced by instrumentation would be added on top of this error. In ddPCR in the low concentration regime, this unavoidable subsampling error is the most significant source of measurement error. More droplets will not change this error: 200 targets in 20,000 droplets and 200 targets in 100,000 droplets (five wells) will lead to the same subsampling error.

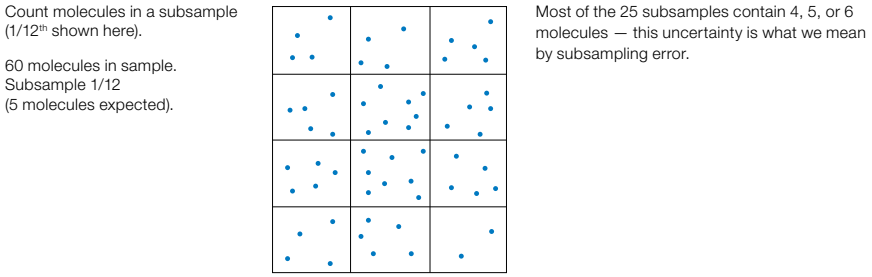

Fig. 1. Subsampling error due to analyzing part of a larger whole.

The error due to subsampling is given as:

$$\text{Standard deviation} = \sqrt{M}$$

$$\text{Coefficient of variation} = \frac{\sqrt{M}}{M}$$

where M = expected number of target molecules in the ddPCR reaction.

Partitioning

The second contribution to the technical error bars comes from partitioning of the DNA targets into droplets. This is the error that dominates at high concentration.

The illustration in Figure 2 shows 288 target molecules partitioned into 144 droplets. These grids are snapshots in time: imagine molecules bouncing around in the sample, then at any given instant divide the sample into 288 partitions. It's evident that most of the partitions do not contain exactly two molecules.

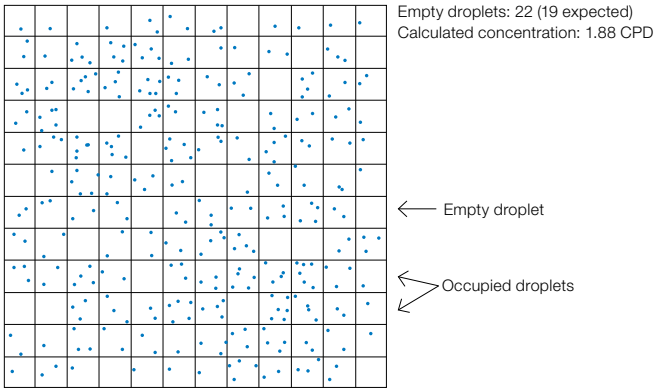

Fig. 2. Target molecule partitioning. CPD, copies per droplet.

Statistics tells us that we should on average see 19 (or more precisely, 19.5) empty partitions or droplets in this experiment. In a particular instant in time, we see 22 empty droplets and we estimate a concentration of 1.88 based on this number. The partitioning in Figure 3 shows different instants in time, and correspondingly different numbers of empty droplets (note that the empty "droplets" are highlighted by a thicker black line). This type of error is governed by Poisson statistics, just like the subsampling error, and is referred to as the "partitioning error."

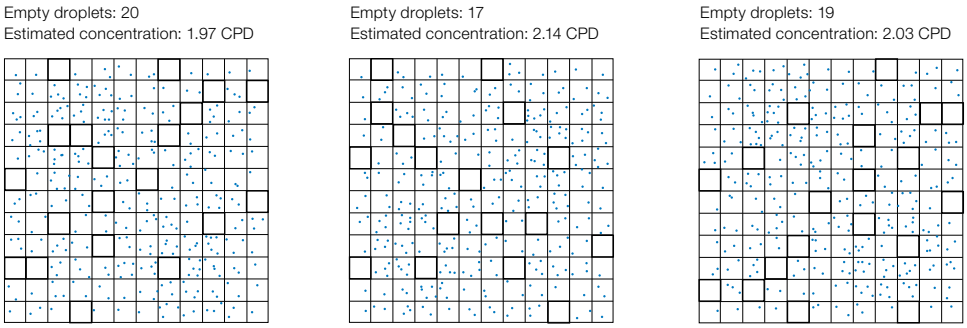

Fig. 3. Target molecule partitioning repeated three times. CPD, copies per droplet.

Figure 4 illustrates the technical error as a function of CPD for 10,000 droplets. Note that the coefficient of variation (CV) is extremely low across the entire range covered here. The dotted lines show the region with CV <2.5%: 0.17–5.1 CPD.

The black and blue curves match very closely at low CPD, meaning that subsampling error is more significant than the partitioning error in this example. The blue curve goes up with high CPD as the contribution of partitioning error increases.

Note: The actual CV calculations contain a Bayesian adjustment at very low and very high CPD (<100 occupied or <100 empty droplets).

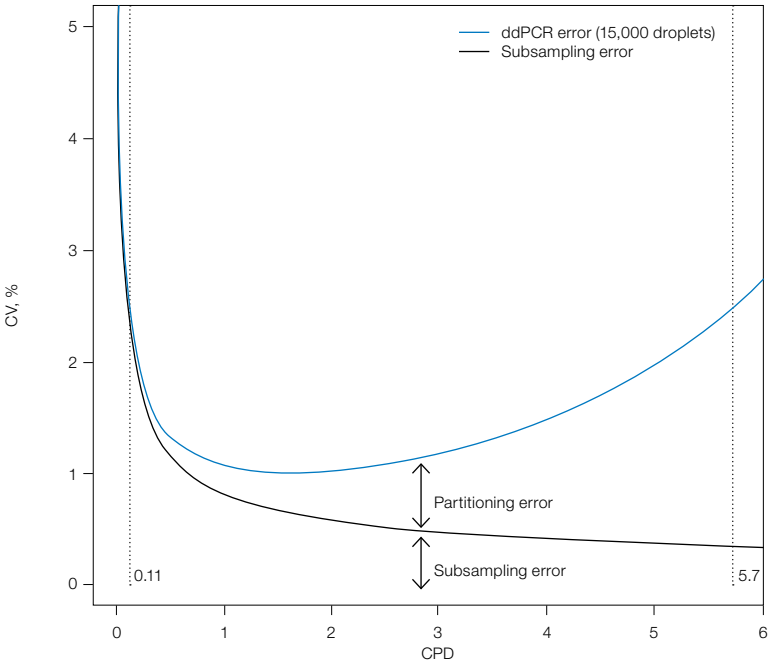

Fig. 4. Relative contribution of partitioning and subsampling errors to ddPCR error. CPD, copies per droplet; CV, coefficient of variation.

Appendix C
Acronyms

Acronyms

ABS — absolute quantification

BLAST — Basic Local Alignment Search Tool

bp — base pair

cDNA — complementary DNA

CN — copy number

CNV — copy number variation

CPD — copies per droplet

CV — coefficient of variation

ddPCR™ — Droplet Digital™ PCR

DNA — deoxyribonucleic acid

dsDNA — double-stranded DNA

dUTP — 2'-deoxyuridine 5'-triphosphate

ERBB2 — human epidermal growth factor receptor 2 gene (also, *HER2*)

FFPE —formalin-fixed, paraffin-embedded

FISH — fluorescence in situ hybridization

GC — guanine-cytosine

gDNA — genomic DNA

GMO — genetically modified organism

HER2 — human epidermal growth factor receptor 2 gene (also, *ERBB2*)

HIV — human immunodeficiency virus

IHC — immunohistochemistry

LNA — locked nucleic acid

LoD — limit of detection

LoQ — limit of quantification

miRNA — microRNA

NGS — next-generation sequencing

NTC — no template control

PBMC — peripheral blood mononuclear cell

PCR — polymerase chain reaction

qPCR — quantitative PCR

RED — rare target sequence detection (rare event detection)

RFU — relative fluorescence units

RMD — rare mutation detection

RNA — ribonucleic acid

RSD — rare sequence detection

RT-ddPCR — reverse transcription Droplet Digital PCR

RT-qPCR — reverse transcription quantitative PCR

SNP — single nucleotide polymorphism

T_M — melting temperature

UDG — uracil DNA glycosylase

UNG — uracil N-glycosylase

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